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LOUIS OTTO KUNKEL, 1884–1960

Francis O. Holmes

The death of Dr. Louis Otto Kunkel, on March 20, 1960, brought to completion a long life devoted to research and administration in plant pathology, principally in the field of virology. At the time of his death Dr. Kunkel was a Member Emeritus of the Rockefeller Institute, where at the age of 75 he was continuing his studies of viral diseases of plants and of the means by which they are transmitted.

The science of virology developed largely during the period of Dr. Kunkel's active research, and he may be said to have influenced it directly or indirectly at almost all stages of its growth. Indeed any account of his lifework inevitably constitutes a history of accomplishment in the field of plant virology. Today many American and foreign institutions where virological investigations are in progress have leaders or staff members drawn from the group that he supervised and trained during his years as pathologist at the Boyce Thompson Institute for Plant Research, Inc., 1923–32, and as head of a group of investigators working in the field of plant pathology at the Rockefeller Institute, 1932–55.

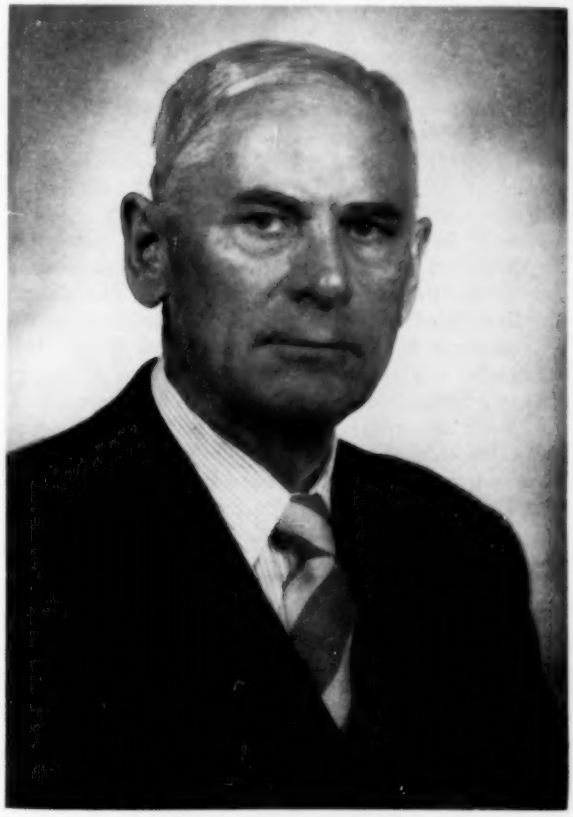
Dr. Kunkel's interest in biology developed early in his life and continued to grow as successive developments of science provided insight into new aspects of the activities of living organisms, especially with respect to microorganisms like fungi and bacteria. After he began to study diseases caused by filterable viruses, he became intensely interested in these and gave many years to their investigation. His belief that viruses represent the smallest of all now recognizable living entities enticed him into protracted studies of those that affected plants, their insect-vector relationships, their transmissibility, their thermal stability, and the entire gamut of their biological relationships as obligate parasites.

His formal education began in Mexico, Missouri. Adjacent to that town was the Kunkel family farm where, through boyhood years, he began to ponder over the manner in which nature expresses herself. His earliest scientific training was received at the University of Missouri, where he matriculated in 1906 and where he obtained the degree of B.S. in 1909 as well as the degree of A.B. in 1910. In a letter to his friend Dr. B. M. Duggar, written in 1950, he acknowledged his debt of gratitude to him for his first introduction to botany during the year 1907.

By 1911 Kunkel had obtained the M.A. degree at the University of Missouri. The following academic year he spent at the Henry Shaw School of Botany of Washington University (at the Missouri Botanical Garden in St. Louis), where he became a candidate for a doctor's degree in botany. The next year he transferred his studies to Columbia University in New York City.

Kunkel's attitudes toward scientific investigations and the publication of scientific findings were greatly influenced by his association with Professor Robert A. Harper, whom he first met in the autumn of 1912 on his arrival at Columbia University as a graduate student. Professor Harper's analyses and criticisms of scientific papers in the lecture classes made a deep impression. Dr. Kunkel wrote later that his own first manuscript received such careful revision that he never prepared a paper for publication afterward without considering what Professor Harper would say about it if it came under his critical eye. This attitude was passed on to many of his later associates. He was never too busy to help them in the preparation of manuscripts or, indeed, in other ways.

In 1914 Kunkel received the degree of Doctor of Philosophy in Botany at Columbia University, his doctoral thesis being concerned with the influence of physical and chemical factors on the toxicity of inorganic salts to the fungus *Neurospora sitophila*. It was in 1915 that he married Johanna Caroline Wortman; their four sons and sixteen grandchildren now carry on the Kunkel family traditions. During most of the period between 1914 and 1920, Dr. Kunkel served as pathologist for the Bureau of Plant Industry of the United States Department of Agriculture, studying especially the powdery scab and wart diseases of potato as well as club root of cabbage. In 1915 and



LOUIS OTTO KUNKEL
1884–1960

1916, however, he held a fellowship that permitted him to travel and study in Europe, at German and Swedish laboratories.

When Dr. Kunkel accepted the position of associate pathologist for the Hawaiian Sugar Planters' Association in 1920, his attention was immediately drawn to the viral diseases of sugar cane and related plants. Within two years he had studied the cell inclusions that were characteristic of corn-stripe disease and had concluded that these might represent some stage of a parasite responsible for causation of the disease. He had found also that the corn leafhopper, *Peregrinus maidis*, could transmit this disease but not the somewhat similar mosaic disease of sugarcane, which he found to be transmitted only by aphids. The inability of leafhoppers to carry aphid-transmissible diseases and of aphids to transmit leafhopper-borne infections was confirmed repeatedly in his later experience.

Dr. Kunkel's return from the Hawaiian Islands coincided with the establishment of the Boyce Thompson Institute for Plant Research, Inc., at Yonkers, N. Y. In preparation for work to be undertaken there, Kunkel visited many American laboratories of plant pathology and related sciences early in 1923. For nine years thereafter he applied information thus gathered to the development of laboratories and a diversified program for the investigation of viral diseases of plants.

Dr. Kunkel himself specialized in studies of the "yellows" type of plant disease. Various phases of this broad subject engaged his active interest for the remainder of his career. At Yonkers he accomplished two principal scientific objectives. The first of these was the discovery that a small gray leafhopper, then known as *Cicadula sexnotata* but now called *Macrosteles fascifrons*, transmitted aster-yellows virus. This work and his subsequent investigations, performed with the thoroughness that characterized his research studies during his entire career, laid the foundation for quantitative studies which he and others subsequently carried out. The second success at Yonkers was the identification of the insect vector of peach yellows as the leafhopper *Macropsis trimaculata*. This constituted a triumphant conclusion of half a century of search for such a vector by a series of investigators.

About 1930, plans of the Rockefeller Institute to add a Division of Plant Pathology to its program of comparative pathology in a branch laboratory located near Princeton in New Jersey led Dr. Simon Flexner and Dr. Thomas M. Rivers to recommend Dr. Kunkel as the man best qualified to head this new project. Dr. Kunkel visited various European laboratories of plant pathology in preparation for the new program of research. Meantime new laboratory and greenhouse facilities were added to the Princeton establishment and in 1932 Dr. Kunkel assembled a group of investigators for the attack on the still mysterious filterable viruses. Seventeen years of research in the Princeton laboratories coincided with remarkably fruitful years in the development of the new science of virology throughout the world. The Princeton group, constantly exchanging its members with staffs of other institutions in America and abroad, contributed much to the advance. Dr. Kunkel himself continued to study new representatives of the yellows group of diseases and demonstrated that many of these could be cured by heat treatments tolerated by the plant hosts.

Integration of the plant pathology laboratories of the Rockefeller Institute at Princeton into the parent laboratories of the Institute in New York City occurred in January 1949. Dr. Kunkel supervised the construction of new laboratory and greenhouse facilities at the New York City location overlooking the East River. He became an Emeritus Member of the Rockefeller Institute staff in July 1949 and in 1955 retired from all his administrative duties, continuing only his own research work.

In the last few years of his life, Dr. Kunkel repeatedly expressed his pleasure that he was able, during the years of his retirement, to add one more substantial finding to the series that had constituted his life-work. This was the demonstration that the insect vector of aster-yellows virus could be protected, through prior feeding on plants carrying one strain of this virus, from subsequent infection by a second strain, just as he had shown earlier that certain plant hosts could be so protected.

Dr. Kunkel's eminence in the field of science was widely recognized. Among other honors, he was elected to membership in the National Academy of Sciences in 1932 and the American Philosophical Society in 1942. He received the Certificate of Merit award at the Golden Jubilee meeting of the Botanical Society of America in 1956 and a Distinguished Service Award of The New York Botanical Garden in 1959. He served as Member of the Board of National Research Fellowships in the Biological Sciences for the National Research Council, as Member of the American Advisory Council for Yenching University at Peiping, China, as Member of the Advisory Council of the Department of Biology at Princeton University, as Member of the Committee to Visit the Division of Biology for the Board of Overseers of Harvard College, and as Member of the Board of Managers and Member of the Scientific Committee for The New York Botanical Garden. His sound judgment and wise counsel were acknowledged with appreciation by the various administrative boards of educational and scientific institutions on which he served.

INHERITANCE OF STEM RUST RESISTANCE OF C.I. 7232, A DERIVED TETRAPLOID OAT

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SUMMARY

Inheritance of stem rust resistance of C.I. 7232, a derived tetraploid oat, was studied. Resistance to stem rust races 7, 7A, and 8 was conditioned by a single dominant gene. The gene controlling resistance to stem rust in C.I. 7232 differs from the genes A, D, BC, and E reported in cultivated hexaploid oats.

The derived tetraploid oat, C.I. 7232 (accession number, Cereal Crops Research Branch, Crops Research Division, ARS, U.S.D.A.), one of the sources of resistance to virulent races of the crown rust fungus (*Puccinia coronata* Cda. var. *avenae* Fraser & Led.), was reported by Simons et al (5) to carry a dominant gene controlling resistance to race 264. Zillinsky et al (7) reported that C.I. 7232 also carries resistance to races 7, 7A, and 8 of the stem rust fungus (*Puccinia graminis* Pers. f. sp. *avenae* Eriks. & E. Henn.). In 1957, routine greenhouse tests of seedlings grown from C.I. 7232 harvested at Aberdeen, Idaho, from 10-foot rows revealed in plant rows 801 and 841 a few plants that were respectively susceptible to races 7A and 8. This paper reports the inheritance of resistance of C.I. 7232 to stem rust races 7, 7A, and 8.

Materials and methods.—C. I. 7232, formerly designated Aberdeen 101, had been self-pollinated for 6 generations and was considered to be a homogeneous line. It is characterized by very early maturity, black kernels, short to medium height, and semi-drooping equilateral panicles. The susceptible and resistant plants of C.I. 7232 cannot be distinguished morphologically but can be differentiated by their reactions to races of the stem rust fungus.

Six F_1 hybrid seeds from reciprocal crosses between susceptible and resistant C.I. 7232 were obtained in a greenhouse at Ames, Iowa, in February, 1957, and were grown at Aberdeen that summer. About 40–50 F_2 seeds were planted in 6-in. pots in a greenhouse. For determination of rust reactions, F_2 seedlings of each cross were divided into 3 groups, and each group was inoculated with a different race of the stem rust fungus. The races were 7, 7A, and 8. The groups of seedlings classified resistant to race 7 or to race 7A were subdivided into 2 subgroups. One subgroup of seedlings resistant to race 7 was tested with race 7A, and the other with race 8. Similarly, the 2 subgroups of seedlings classified as resistant to race 7A were tested, one with race 7 and the other with race 8. Rust reactions were determined on seedlings in the first- and second-leaf stages and were classified on the basis of infection types 0 to 4, with 0, 1, and 2 considered resistant and 3 and 4 susceptible.

Results.—High-type resistance characterized by infection types 0 to 1+ was observed on all the resistant plants, and infection types 3 to 4 on all susceptible plants. Parental plants resistant to race 8 were also resistant to races 7 and 7A, and plants susceptible to race 8 were also susceptible to 7A and 7 (Table 1). Backcross lines isogenic for stem rust genes A, BC, and D, and a homozygous recessive line abcd, were tested (Table 1).

The segregation of the F_2 seedlings in 6 crosses for reaction to races 7, 7A, and 8 is presented in Table 1. In all crosses, the observed segregation for reaction to race 7 is in agreement with the hypothesis of a single dominant gene controlling resistance. Ratios that did not fit the expected 3:1 segregation of a single-gene difference were observed for respective reactions to races 7A and 8 in crosses 2 and 1. Segregation for reaction to races 7A and 8 in the remaining crosses was in agreement with a single dominant gene hypothesis.

Table 1.—Reactions of P_1, P_2, and F_2 populations of oat crosses between susceptible and resistant C.I. 7232 to stem rust races 7, 7A, and 8, and chi-square goodness of fit to a 3:1 ratio.

	Reaction of F_2 plants, parents and testers								
	Race 7			Race 7A			Race 8		
Crosses, parents, and testers	Resist.	Susc.	P value	Resist.	Susc.	P value	Resist.	Susc.	P value
1. C.I. 7232 (R)[a] × C.I. 7232 (S)[a]	97	35	.50–.70	103	23	.05–.10	13	12	.01
2. C.I. 7232 (R) × C.I. 7232 (S)	100	38	.30–.50	85	15	.02–.05	16	6	.80–.90
3. C.I. 7232 (S) × C.I. 7232 (R)	67	25	.50–.70	60	15	.30–.50	19	8	.50–.70
4. C.I. 7232 (S) × C.I. 7232 (R)	80	26	.90–.95	82	26	.80–.90	10	4	.70–.80
5. C.I. 7232 (S) × C.I. 7232 (R)	77	22	.50–.70	82	26	.80–.90	74	26	.80–.90
6. C.I. 7232 (S) × C.I. 7232 (R)	72	31	.20–.30	83	28	.80–.90	73	26	.70–.80
C.I. 7232 (S)	0	33		0	39		0	17	
C.I. 7232 (R)	36	0		36	0		13	0	
Backcross line Abcd	15	0		27	0		0	20	
Backcross line aBCd	28	0		0	29		25	0	
Backcross line abcD	0	20		0	23		20	0	
Recurrent line abcd	0	14		0	24		0	18	

[a] R and S respectively denote resistance and susceptibility to stem rust races 7, 7A, and 8.

Table 2.—Reaction of F_2 seedlings resistant to either race 7 or 7A after inoculation with two additional races.

Race to which initially resistant, and cross	Plants in indicated group when tested with indicated race					
	7		7A		8	
	Resist.	Susc.	Resist.	Susc.	Resist.	Susc.
Race 7:						
1. C.I. 7232 (R)[a] × C.I. 7232 (S)[a]			31	2	43	2
2. C.I. 7232 (R) × C.I. 7232 (S)			32	5	32	1
3. C.I. 7232 (S) × C.I. 7232 (R)			35	3	24	3
4. C.I. 7232 (S) × C.I. 7232 (R)			41	1	40	2
Race 7A:						
1. C.I. 7232 (R) × C.I. 7232 (S)	46	8			42	0
2. C.I. 7232 (R) × C.I. 7232 (S)	35	10			33	0
3. C.I. 7232 (S) × C.I. 7232 (R)	30	5			23	5
4. C.I. 7232 (S) × C.I. 7232 (R)	34	6			36	5
5. C.I. 7232 (S) × C.I. 7232 (R)	39	1			38	2
6. C.I. 7232 (S) × C.I. 7232 (R)	38	0			42	1

[a] R and S respectively denote resistance and susceptibility to stem rust races 7, 7A, and 8.

Seedlings classified as resistant to race 7 or 7A were tested with two other races of the stem rust fungus (Table 2). The reactions of the plants to the two additional races were similar to their reactions to race 7 or 7A except that a few were susceptible. Plants showing susceptible reactions in the second test were probably "escapes," i.e., susceptible plants that failed to develop adequate symptoms after the initial inoculation with race 7 or 7A because of unfavorable environment. If the seedlings classified susceptible in the second test can be assumed to be "escapes," it is possible to correct the number of resistant and susceptible plants observed in the initial test with races 7 and 7A. Such a correction was applied to the original data, and Table 3 presents corrected ratios for reactions to races 7 and 7A and their chi-square P values. Agreement with a hypothesis that a single dominant gene controls resistance to race 7 was not changed in crosses 1, 2, 3, and 4 with the correction applied to the initial data. For reaction to race 7A, however, P values denoting a good and a poor fit to a 3:1 ratio were respectively obtained in crosses 2 and 4.

Discussion.—The data and chi-square analyses indicated that resistance to races 7, 7A, and 8 was conditioned by a single dominant gene. Additional tests with races 7 and 8 and 7A and 8 on seedlings respectively tested originally with races 7A and 7, indicated that resistance to all 3 races was governed by the same gene. The small number of seedlings showing susceptible reactions in the second test was apparently due to misclassification of "escapes" as resistant plants in the initial test.

The gene controlling resistance in C.I. 7232 differs in its reaction to races 7, 7A, and 8 from the A, BC, D, and E genes reported in the hexaploid species. Gene A confers resistance to races 7 and 7A, gene BC to races 7 and 8, gene D to race 8, and gene E to some races other than the three used in the present investigation (6). C.D. 3820, a parent of C.I. 7232, has two independent dominant genes controlling resistance to stem rust races 6, 7, 7A, and 8 (4). Zillinsky et al (7) reported that the reaction of Abd. 101 (C.I. 7232) to 2 isolates of race 6 seemed to indicate that the gene or genes controlling resistance to stem rust races 6, 7, 7A, and 8 could have come from varieties other than its parents.

How did the plants susceptible to races 7A and 8 originate in C.I. 7232, which was considered true-breeding for stem and crown rust resistance? Among the possible means by which susceptible plants could have appeared in the line are natural cross-pollination in the field, mutation, seed contamination, loss of the chromosome or chromosome segment on which is the gene controlling resistance, and breakage of a refractory linkage in the repulsion phase. Among these possibilities, natural crossing is viewed as the most likely. Coffman and Wiebe (2), Konzak (3), and Caldecott et al (1) reported instances of natural crossing in hexaploid oats. Caldecott et al indicated the necessity of isolation in their studies of mutations induced

Table 3.—Corrected F_2 segregation ratio and goodness to fit to a 3:1 ratio of crosses between stem-rust-resistant and stem-rust-susceptible C.I. 7232.

Cross	Race 7			Race 7A		
	Resist.	Susc.	P value	Resist.	Susc.	P value
1. C.I. 7232 (R)[a] × C.I. 7232 (S)[a]	93	39	.20–.30	95	31	.90
2. C.I. 7232 (R) × C.I. 7232 (S)	94	44	.05–.10	73	27	.50–.70
3. C.I. 7232 (S) × C.I. 7232 (R)	61	31	.05–.10	50	25	.05–.10
4. C.I. 7232 (S) × C.I. 7232 (R)	77	29	.50–.70	71	37	.02–.05
5. C.I. 7232 (S) × C.I. 7232 (R)				79	29	.50–.70
6. C.I. 7232 (S) × C.I. 7232 (R)				82	29	.50–.70

[a] R and S respectively denote resistance and susceptibility to stem rust races 7, 7A, and 8.

in populations derived from X-irradiated seeds. In the ordinary field procedure of increasing seed supply, growing of varieties in isolation is not a regular practice. Consequently, cross-pollination, although infrequent, must be considered one of the more likely explanations of the origin of the susceptible plants in the homogeneous line. Data or information now available does not exclude other avenues by which susceptible plants can arise in a resistant line.

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TOXICITY OF ACETIC ACID TO CLADOSPORIUM CUCUMERINUM

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SUMMARY

Acetic acid incorporated into a semi-synthetic liquid medium at 0.25*M* was fungicidal to *Cladosporium cucumerinum* at pH 6.1 and below. The same basal medium containing 0.01*M* acetic acid supported good growth of the fungus at pH 6.0 but was fungicidal at pH 4.0. A similar pattern of toxicity was observed with either propionic or butyric acids but not with fumaric, oxalic, tartaric, succinic, or malic acid. Acetic acid at low pH values also inhibited the growth of 5 other species of fungi: *Fusarium oxysporum* f. *batatas*, *Pythium aphanidermatum*, *Pellicularia filamentosa*, *Aspergillus flavus*, and *Colletotrichum lagenarium* race 1. Spores of one of these fungi, *A. flavus*, failed to grow in the liquid medium at the lower pH values but remained viable.

Introduction.—Nutritional studies involving acetic and other fatty acids include the work of Schade (6), who found that *Leptomitus lacteus* (Roth) Agardh, a fungus that does not utilize sugars, grew on acetic, butyric, and capric acids but not on formic or propionic acids. *Aspergillus niger* van Tiegh. grew on acetate, lactate, tartrate, malate, and fumarate (7). Machlis (4) found that acetate was equivalent to glucose as a carbon source for the growth of *Allomyces javanicus* Kniep. Jarvis and Johnson (3) reported that the use of acetate by *Penicillium chrysogenum* Thom was strongly dependent on pH. Acetate was utilized in fat synthesis by yeasts, but not below pH 5 with *Saccharomyces cerevisiae* Meyen (10, 11). Fencl and Leopold (1) reported a similar pattern of acetate inhibition with *A. niger*. Growth of the fungus was inhibited by acetate at pH 4.2 but not at pH 7.0. Inhibition of growth was further demonstrated with low concentrations of certain other fatty acids. Samson et al (5) have shown that acetate and other short-chain fatty acids inhibited phosphate uptake and accumulation as well as respiration of *S. cerevisiae* at a pH of 4.5. This inhibition was alleviated when yeast cells were washed. The authors suggested that the effect of acetate was probably a direct result of the interaction of the fatty acids or anions with certain enzymes of the yeast. Fencl and Leopold (2) demonstrated respiratory inhibition in *A. niger* with acetic acid. This inhibition was also found to be reversible by raising the pH from 4.2 to 5.5. These authors disagreed with Samson et al (5) on the site of inhibition, stating that undissociated acetic acid inhibited spore germination and that the site of inhibition was at the cell surface.

Strider and Winstead (9) reported that *C. cucumerinum* Ell. & Arth. grew well at pH values below 6.0 on a basal medium containing 1.5% citric acid but not on the same basal medium containing 1.5% acetic acid. It was apparent in these studies that acetic acid or some compound formed by acetic acid in the basal medium was extremely toxic to growth of the pathogen.

The tests reported herein were designed to determine: 1) whether acetic acid or some compound formed by acetic acid in the basal medium was the toxic principle; 2) whether acetic acid was also toxic to mycelium of the fungus; 3) how rapidly the toxic principle acted on spores of *C. cucumerinum;* 4) whether certain other organic acids were toxic to spores of *C. cucumerinum;* 5) the toxicity of several concentrations of acetic acid at various pH levels; and 6) whether acetic acid was also toxic to other fungi. The results have been abstracted (8).

Materials and methods.—The basal medium used was: bacto-casamino acids (1.0 g), K_2HPO_4 (4.0 g), $MgSO_4$ (0.1 g), $FeCl_3$ (0.02 g), sucrose (10.0 g), glycine (6.0 g), and distilled water (1.0 L). Normal HCl or NaOH was added to obtain the various pH levels. Unless otherwise indicated sterilization was

Table 1.—Toxicity of certain organic acids at various pH values to the growth of *C. cucumerinum*.

Organic acid	pH	Growth[a] at stated molarity				
		0.17	0.08	0.04	0.01	0.005
Acetic	3.0	N	N	N	N	
	4.0	N	N	N	N	
	5.0	N	N	N	Y	
	6.0	N	Y	Y	Y	
Propionic						
	3.0		N		N	
	4.0		N		N	
	5.0		N		N	
	6.0		Y		Y	
Butyric						
	3.0		N		N	
	4.0		N		N	
	5.0		N		N	
	6.0		N		Y	
Acetic, propionic, and butyric						
	3.0			N	N	N
	4.0			N	N	N
	5.0			N	N	N
	6.0			N	Y	Y

[a] (Y) = growth present; (N) = growth absent; blanks indicate "not tested."

by autoclaving 8 minutes at 123°C. Fungi were grown in still, liquid cultures in 40 ml of medium contained in 125-ml Erlenmeyer flasks. Spores for use as inoculum were obtained from 5-day-old PDA cultures. The spore concentration was adjusted so that the final spore concentration was about 10^3 spores per ml of medium. All tests were conducted at about 24°C. Other methods are indicated along with the results of the particular experiment.

Results.—*Determination of toxic substance.*—To determine the toxic compound, the basal medium containing 1.5% acetic acid was altered in several different ways, maintaining the same concentrations of the constituents. Each organic ingredient was omitted in turn; KNO_3 was substituted for casamino acids; citric acid was substituted for acetic acid; and the resulting media were tested for toxicity to *C. cucumerinum*. In addition glycine and acetic acid were tested individually and in combination. Media in all tests were adjusted to 3 pH values: 4, 5, and 6. A distilled water blank was also tested. Each treatment was replicated 3 times. The media were autoclaved, seeded with a spore suspension, and incubated 6 days. Growth was observed on all treatments except the water blank and media containing acetic acid. Transfers to PDA from the media containing acetic acid were not viable, though transfers from the water blanks were.

The basal medium containing 0.25*M* acetic acid adjusted to both pH 5.5 or 6.0 was sterilized by Seitz filtration. Forty ml of medium was placed in each of six 125-ml flasks, seeded with a spore suspension, and incubated 8 days. No growth was observed on the Seitz-sterilized media containing acetic acid, and transfers to PDA produced no growth after additional incubation.

Speed of acetic acid toxicity.—The basal medium containing 0.25*M* acetic acid was adjusted to pH 3.7 and 5.6 and seeded as before. Transfers to PDA were made after exposure in these media for 1, 10, 30, 60, and 120 minutes. Each treatment was replicated 4 times. Incubation on PDA was at 24°C for 8 days. At pH 3.7, spores subjected to the 0.25*M* acetic acid medium for one minute or longer made no growth when transferred to PDA. At pH 5.6, spores of the pathogen were viable after all time intervals except for the 120-minute interval.

Determination of exact pH of acetic acid toxicity.—To determine more precisely the pH at which 0.25*M* acetic acid was toxic to the organism, a study was initiated to determine toxicity between pH 5.8 and 6.3. Materials and methods were identical to those previously described except that 0.1*M* K_2HPO_4 was used to stabilize the pH values. To prevent excessive dilution, 4.0*M* NaOH was used to adjust pH. The acidity of the medium was adjusted before autoclaving, checked after, and rechecked 4 and 8 days after seeding. Four flasks were seeded and 2 were retained as controls on the pH of the unseeded medium. The pH of the medium was not changed by autoclaving. The fungus failed to grow at pH 6.1 or below. Inhibition of growth was marked at pH 6.2, but growth was moderate at 6.3. The pH values of the medium remained constant during incubation except where growth occurred. Growth of the fungus tended to raise pH slightly. This test was repeated with identical results.

Tests on the toxicity of other acetates.—Sodium, calcium, and ammonium acetate at 0.25*M* were substituted individually for 0.25*M* acetic acid in the basal medium to determine whether these compounds were also toxic to the fungus. Media were adjusted to 4 pH values: 5.0, 5.5, 6.0, and 6.5. Observations were made after 4, 8, and 12 days at 24°C. The acetate-containing media shifted to slightly lower pH values upon autoclaving. The fungus grew on all media with pH values above 6.0, but on only one below this value. The medium containing sodium acetate at pH 6.0 supported slight growth, though no growth occurred on this medium at pH 5.5.

Effect of certain other organic acids on the growth of C. cucumerinum.—Preliminary tests had indicated that 0.5% acetic acid was toxic to the organism at pH 5.0 but not at 6.0, and that 1.5% acetic acid was toxic at both pH values. Therefore 1.5% DL-malic, succinic, tartaric, oxalic, fumaric, propionic, and butyric acids were tested, with and without 0.5% acetic acid. Two pH values, about 5.0 and 7.0, were used for each treatment. Each pH value of each treatment was replicated 5 times. Observations were made after 4 and 8 days of incubation. After autoclaving the pH values of the media used in this test were 5.1 ± 0.1 and 7.0 ± 0.3. Growth occurred at the higher pH value in every treatment. When 0.5% acetic acid was used, however, no growth was observed at the lower pH value. Growth did occur at the lower pH value in the absence of acetic acid in all treatments except those containing propionic or butyric acids. In flasks where the fungus did not grow, pH remained essentially unchanged:

Table 2.—Effect on the growth of several fungi of acetic acid in the basal medium at various pH values.

Fungus	pH	Acetic acid concentration 0.25*M*	0.17*M*
F. oxyporum f. *batatas*	4.0	N*	N
	5.0	N	N
	6.0	Y	Y
	7.0	Y	Y
A. flavus	4.0	N	N
	5.0	N	Y
	6.0	Y	Y
	7.0	Y	Y
P. aphanidermatum	4.0	N	N
	5.0	N	N
	6.0	N	N
	7.0	Y	Y
P. filamentosa	4.0	N	N
	5.0	N	N
	6.0	Y	Y
	7.0	Y	Y
C. lagenarium race 1	4.0	N	N
	5.0	N	N
	6.0	N	Y
	7.0	Y	Y
C. cucumerinum	4.0	N	N
	5.0	N	N
	6.0	N	N
	7.0	Y	Y

* (N) = growth absent; (Y) = growth present.

when the fungus grew, the media became more basic. The pH of the uninoculated media remained constant during the test period. The fungus did not grow when transfers were made to PDA plates from flasks in which no growth had occurred.

Effect on toxicity of C. cucumerinum of lower concentrations and pH values of 3 organic acids.—Since 1.5% acetic, propionic, and butyric acids were toxic to the fungus at pH 5.0, these acids were tested for toxicity at lower concentrations and at several pH values. The concentrations used were: acetic, 0.17, 0.08, 0.04, and 0.01*M*; propionic, 0.08 and 0.01*M*; butyric, 0.08 and 0.01*M*; and a combination of acetic, propionic, and butyric at 0.04, 0.01, and 0.005*M* each. Media for each treatment were adjusted before autoclaving to 4 pH levels—3, 4, 5, and 6. Each treatment was incubated 8 days as previously described. Growth of the pathogen was not observed on media at the lowest concentrations of organic acids tested at the lowest pH level used (Table 1). Propionic and butyric acids at $10^{-2}M$ were toxic to the fungus at pH 5.0 but not at 6.0. Acetic acid at $10^{-2}M$ prevented growth of the organism at pH 4.0 but not at 5.0. No growth developed in transfers to PDA from media on which the fungus failed to grow.

Effect of acetic acid on the growth of mycelial mats of C. cucumerinum.—*C. cucumerinum* was grown for 6 days at 24°C on a Richard's solution containing 1.0 g of casamino acids per liter of medium. After 6 days, mycelial mats were placed in flasks with 40 ml of the basal medium containing 0.25*M* acetic acid. The pH was adjusted to 5.5, and the treatment was replicated 8 times. After 36, 72, and 108 hours, the mycelial mats were removed, washed in sterile distilled water, and placed on PDA in Petri dishes. Observations on viability or resumption of growth were made 8 days after PDA transfers. No further growth was observed after mycelial mats had been placed in the medium containing acetic acid, and no transfers to PDA resumed growth.

Effect of acetic acid on certain other fungi.—Growth of the following fungi were tested on the basal medium plus 0.17 and 0.25*M* acetic acid at pH values of 4, 5, 6, and 7: *Fusarium oxysporum* f. *batatas* (Wr.) Snyd. & Hans., *Aspergillus flavus* Lk., *Pythium aphanidermatum* (Edson) Fitz, *Pellicularia filamentosa* (Pat.) Rogers, and *Colletotrichum lagenarium* (Pass.) Ell. & Halst. race 1 Goode. *C. cucumerinum* was used for comparison. Growth of the fungi was also tested with 0.08*M* citric acid at the same pH values. Inoculation was by spore suspension in all cases except with *P. aphanidermatum* and *P. filamentosa*, when mycelium was the inoculum. Observations were made after 8 days of incubation.

All fungi tested grew well on the medium containing citric acid at all pH values but one. *C. lagenarium* (Race 1) failed to grow at pH 7.0 but grew at all other pH values. In general, as with *C. cucumerinum*, these various fungi grew in the acetic-acid-containing medium at the higher pH values but failed to grow at certain lower pH values (Table 2). No growth developed in transfers to PDA from media on which *C. cucumerinum* failed to grow. Such transfers from media on which *A. flavus* failed to grow did reveal resumption of growth in every case. The other test fungi in this investigation were not transferred to PDA.

Discussion.—Acetic acid at $10^{-2}M$ was toxic to the organism at the pH optimum for growth of the fungus. Propionic and butyric acids at $10^{-2}M$ were also toxic at the pH optimum for growth of *C. cucumerinum* and at slightly higher pH levels. In all tests the concentrations of acetic acid were somewhat lower than that reported, because of loss of this volatile material during autoclaving.

Table 3.—Growth inhibition of *C. cucumerinum* by molecular acetic acid.

Total *M* conc. of acetic acid plus acetate	pH	*M* conc. of acetic acid (approximate)	Inhibition
.25	5.0	.0833	Complete
.04	4.0	.0332	Complete
.08	5.0	.0237	Complete
.04	5.0	.0133	Complete
.25	6.0	.0125	Complete
.25	6.1	.0100	Complete
.17	6.0	.0085	Complete
.01	4.0	.0083	Complete
.25	6.2	.0075	Strong
.25	6.3	.0063	Moderate
.08	6.0	.0040	Slight
.07	5.0	.0033	Slight
.04	6.0	.0020	None
.25	7.0	.0013	None

By calculating the concentration of molecular acetic acid at the various pH levels used in the above tests, it may be shown that acetic acid is the toxicant to spores of *C. cucumerinum*, not acetate ions (Table 3). A comparable table for butyric and propionic acids would show that these molecules are similarly toxic to *C. cucumerinum* spores, not their respective cations. Fencl and Leopold (1) found that propionic and butyric acids were inhibitory to spores of *A. niger*, even at pH 7.0. Since those authors observed inhibition at pH 7.0 with *A. niger* and since they found that acetic acid inhibition was alleviated by an increase in pH, the mechanism of toxicity of these fatty acids to *A. niger* was different from that to *C. cucumerinum*. No reversibility of inhibition, as reported for *A. niger* (2) or for *S. cerevisiae* (5), was observed with *C. cucumerinum*. Reversibility was definitely demonstrated with spores of *A. flavus*, however. Fencl and Leopold and Samson et al are not in agreement as to the site at which fatty acids have their effect. The former said the effect is at the cell surface (2), not within the cell, as suggested by Samson et al (5). The present work indicates that the site may differ with different fungi. Our results with *C. cucumerinum* tend to favor the site within the cell, whereas our results with *A. flavus* tend to favor the site at the cell surface.

Future studies on the mechanism(s) of resistance of cucumber to the scab organism should include qualitative and quantitative analyses of the short-chain fatty acids of resistant and susceptible cucumber tissue. The growth of the organism on the fatty acid fractions of expressed sap of resistant and susceptible cucumber tissue should be investigated concurrently.—North Carolina State College, Raleigh.

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EFFECT ON ROOT-KNOT NEMATODE POPULATIONS OF RESISTANT AND SUSCEPTIBLE COTTON

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SUMMARY

The population levels of *Meloidogyne incognita acrita*, *Pratylenchus brachyurus*, *Criconemoides ornatum*, *Trichodorus christiei*, *Hoplolaimus tylenchiformis*, and various saprophagous nematodes were determined under field conditions in the root zone of 5 cotton varieties and selections having a wide range of root-knot-nematode resistance. Larval populations of *M. incognita acrita* increased slowly under the more resistant cottons, such as a selection of *Gossypium barbadense* and a selection and variety of *G. hirsutum* (a Mexican selection and Auburn 56 variety). The increase was more rapid under Empire and Rowden, susceptible varieties of *G. hirsutum*. In September, however, when the populations of this nematode were at the highest level under all varieties, no appreciable differences existed between populations under the resistant and the susceptible varieties. No correlation was apparent between root-knot resistance and population levels of *T. christiei*, *P. brachyurus*, and *C. ornatum*. Populations of *H. tylenchiformis*, however, were considerably lower under *G. barbadense* than under other entries. These low population levels may be indicative of host unsuitability. Saprophagous nematodes occurred in relatively high numbers throughout the season. There were no indications that any one cotton tested was more conducive to their development than another.

Introduction.—Root-knot-nematode-resistant varieties have long been used to combat damage to crops in infested soil (5). Atkison (2) reported as early as 1889 that cotton is severely damaged by root-knot nematodes. From tests with several cotton varieties, Miles (4) and Smith (7, 8, 9, 10) reported a range of susceptibility from moderate to complete, and Smith (10) reported that Auburn 56 was superior in both root-knot resistance and the correlated Fusarium resistance. A search (1) has been made through the species, subspecies, and numerous varieties of cotton for a better source of root-knot resistance. Thus far,

the most promising material found has been a wild selection of *G. barbadense* Linn. This selection was formerly thought to be *G. barbadense* var. *darwinii*, but the authors have been advised (personal correspondence with T. Kerr, Agronomist) that the selection is not a variety.

The most resistant variety of upland cotton has much less than complete resistance, as determined by the root-knot index method (9). This partial resistance has unknown effects on the population dynamics of root-knot nematodes and other nematode species under field conditions. Sauer and Giles (6) reported that growing hybrid tomatoes (*Lycopersicon esculentum* and *L. peruvianum*) resistant to *Meloidogyne javanica* assured good yields but did not reduce the nematode population enough to make the land suitable for susceptible crop plants. Since resistance varied from plant to plant, some reproduction of the nematodes occurred. Clayton et al (3) reported that corn, though rarely showing root-knot damage, does not actually reduce root-knot nematode populations. Smith (9) reported field observations indicating that some improvement in resistance to other nematode species could occur with improved root-knot resistance.

This investigation was conducted to determine the effects of root-knot-resistant and susceptible cotton varieties on a) the root-knot nematode larval population of field soil and b) the population of other nematode species present.

Materials and methods.—The experiment was conducted at the Plant Breeding Unit, Tallassee, Alabama, in an area where cotton had been grown continuously for several years. The soil had natural populations of *Meloidogyne incognita acrita* Chitwood, 1949; *Pratylenchus brachyurus* (Godfrey, 1929) Filipjev & Schuurmans-Stekhoven, 1941; *Criconemoides ornatum* (Kir'ia-nova, 1948) Raski, 1958; *Trichodorus christiei* Allen, 1957; *Hoplolaimus tylenchiformis* Daday, 1905; and various saprophagous nematodes. The cotton wilt fungus, *Fusarium oxysporum* Schlecht. *f. vasinfectum* (Atk.) Snyd. & Hans., was also present. The 5 types of cotton used, with their root-knot indices (unpublished data), were: a wild selection of *G. barbadense*, 34; a Mexican selection of *G. hirsutum* Linn., 51; and three varieties of *G. hirsutum* (Auburn 56, 67; Empire, 85; and Rowden, 98).

Delinted thiram-treated seeds of each variety were planted on April 16, 1958, in rows 30 ft long and 40 in. apart, replicated 4 times. From emergence until mid-July the soil was cultivated frequently for weed control. Dieldrin was applied to the plants 12 times for insect control. At planting time 400 lb/A. of 4-12-12 fertilizer was applied. On June 1 the plants received a side-dressing of 200 lb/A. of ammonium nitrate.

At monthly intervals (May–October), nematode counts were obtained from soil samples taken with an 8-in. soil sampling tube from all replications of each variety. Borings were made in the root zone 6 in. from the base of the plants. The soil was placed in plastic bags and taken immediately to the laboratory for processing. Nematodes were recovered from 1-pint

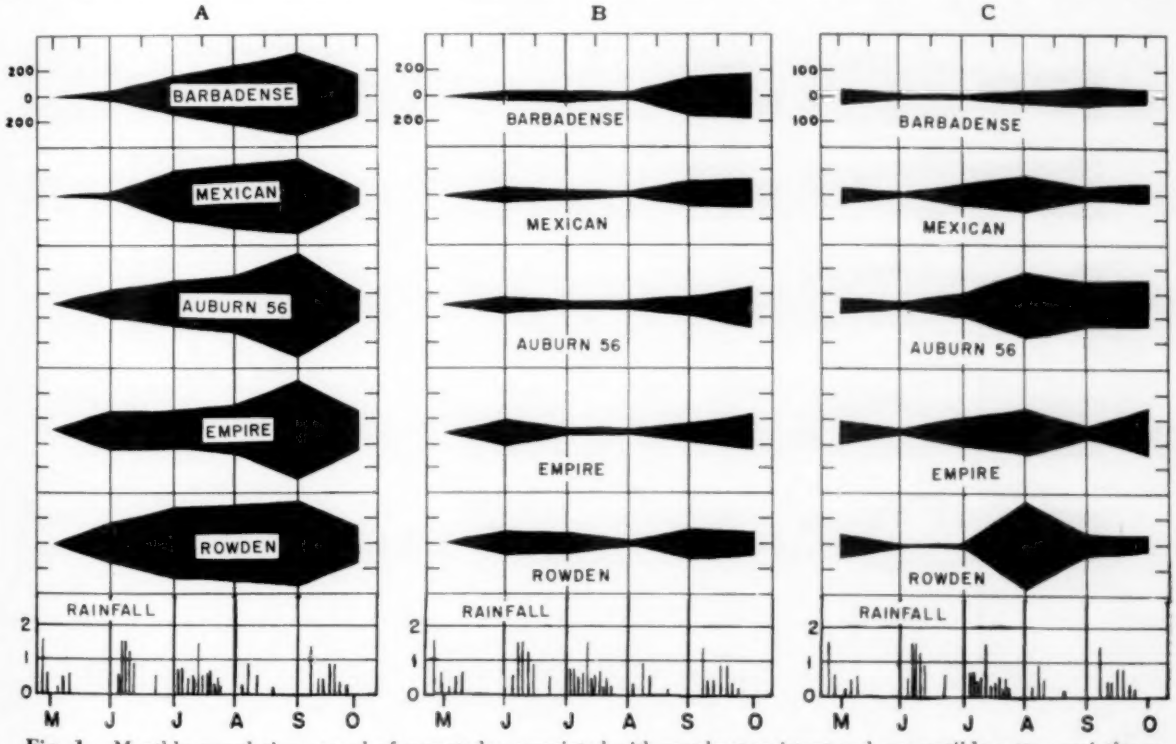

Fig. 1.—Monthly population record of nematodes associated with root-knot-resistant and -susceptible cotton varieties reported in terms of nematodes per pint of soil: **A)** *Meloidogyne incognita acrita*, **B)** *Trichodorus christiei*, **C)** *Hoplolaimus tylenchiformis*.

portions of the samples with aid of Seinhorst elutriators. Rainfall during the experiment was recorded daily.

Results.—Fig. 1 and 2 show the average numbers of the various nematodes present on each collection date per pint of soil from each cotton variety. Nematode populations are represented by the shaded areas in the charts. Specific populations may be calculated by combining the intervals above and below the zero axis. For example, in Fig. 1-A the number of nematodes in September for *G. barbadense* is 600. It should be noted that the scale used in the graphs is not the same for all nematode genera. Daily rainfall is expressed in inches at the bottom of each figure.

The numbers of *M. incognita acrita* larvae were at very low levels under all cotton varieties in May, but by June had increased greatly under the two susceptible varieties, Empire and Rowden. Increases were only slight under the two highly resistant entries, *G. barbadense* and the Mexican selection. The increase under Auburn 56, a moderately resistant variety, was intermediate. The changes in population levels were perhaps the result of differential rate of reproduction on the varieties, since time from planting date was sufficient to permit hatching of the first generation. The larval population under the Rowden variety rose to a high level in June, remained almost constant in July and August, and fell sharply in September. Rowden plants grew well in the early part of the season, but by July many were dying from the nematode-wilt complex. By October all Rowden plants were dead. Plants of the other varieties and selections were still alive, though growth had become slower. Larvae under all cottons increased in numbers through August. In September, when the numbers of root-knot larvae were greatest under all varieties, Auburn 56 ranked highest in larval counts, Empire second, *G. barbadense* third, Rowden fourth, and the Mexican selection fifth. Numbers of larvae had declined under all entries by October.

The trend of *T. christiei* was essentially the same under all varieties and selections throughout the experiment: low but increasing in May, declined in June and July, and increased again in August and September. These nematodes were more abundant under the root-knot-susceptible varieties in June, and under *G. barbadense* and Auburn 56 in October.

H. tylenchiformis increased considerably less under *G. barbadense* than under the other plants which may indicate differences in host suitability. *H. tylenchiformis* differed from the other nematodes in two respects: It had the highest population level of any of the parasitic species on the first sampling date, and its highest population level was in July instead of August and September, as with the other species.

The populations of *P. brachyurus* were rather low throughout the experiment. The differences under different plants were too small to permit conclusions on plant influence. No attempt was made to determine the number of nematodes present in the roots.

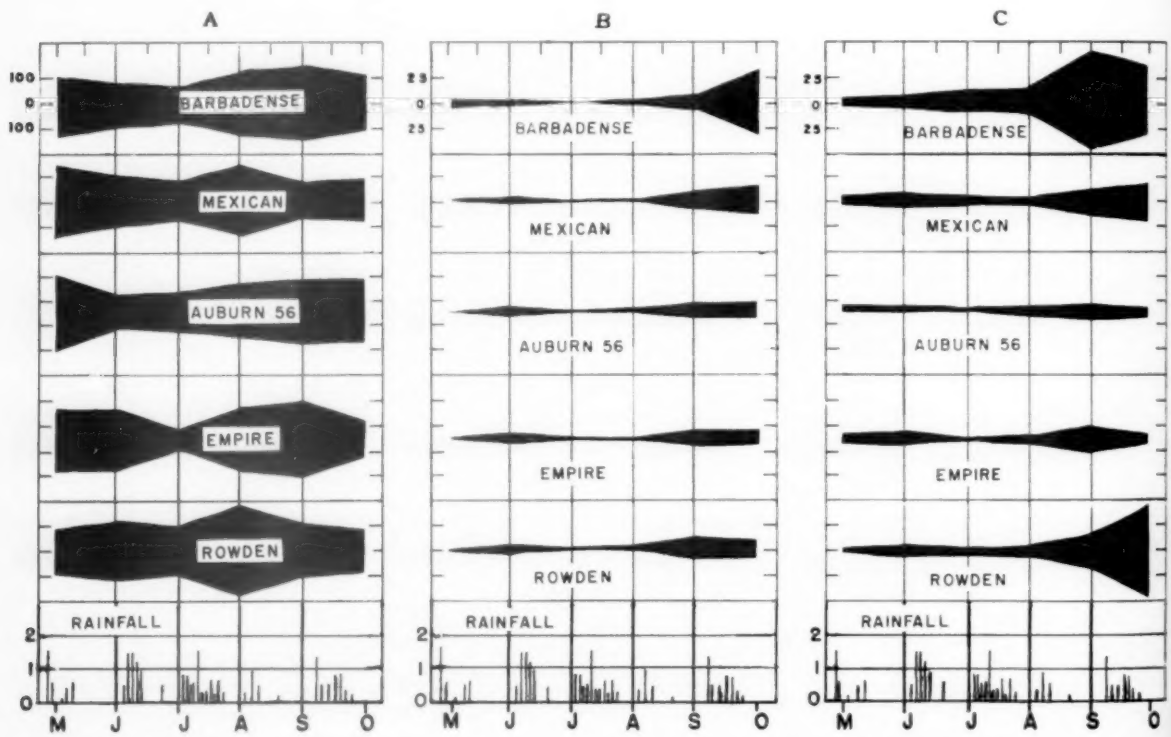

Fig. 2.—Monthly population record of nematodes associated with root-knot-resistant and -susceptible cotton varieties reported in terms of nematodes per pint of soil: **A)** Saprophagous nematodes, **B)** *Pratylenchus brachyurus*, **C)** *Criconemoides ornatum*.

C. ornatum numbers were low under Empire, Auburn 56, and the Mexican selection throughout the experiment. Only a few were present under Rowden and *G. barbadense* early in the season, but numbers were large in September and October.

Saprophagous nematodes occurred in relatively high numbers under all cotton varieties and selections throughout the experiment. No single variety appeared to be more conducive to their development than another.

In no case did rainfall and nematode populations appear closely correlated. *H. tylenchiformis* followed rainfall trends more closely than the other species. Apparently no critical moisture shortage occurred in the soil during the experiment for any of the nematodes, except possibly *H. tylenchiformis*.

Discussion.—Under field conditions, presently available root-knot-resistant cotton varieties and selections may be expected to maintain root-knot larval populations equal to or even greater than those maintained by susceptible ones. Resistant cotton, therefore, cannot be relied on to reduce root-knot nematode infestation in a rotation schedule. Resistance as used in this paper is measured in terms of numbers and size of root-knot galls. The severity of galling is expressed as root-knot indices, indicating host response. The root-knot indices obtained for *G. barbadense*, Mexican selection, and Auburn 56 are comparatively low although these cottons maintained high root-knot larval populations. The seeming paradox may be due in part to the more extensive root systems of the resistant plants. Nematode and *Fusarium* wilt injury causes more root deterioration of susceptible plants than of resistant plants, thereby reducing the total amount of roots available for root-knot nematode reproduction.

There was no strong evidence of correlations between root-knot resistance and population responses of *T. christiei*, *P. brachyurus*, and *C. ornatum*. The low population levels of *H. tylenchiformis* under *G. barbadense*, however, suggest host unsuitability. From the standpoint of numbers of nematodes that developed during the period of plant growth and boll development, *T. christiei* and *H. tylenchiformis* appeared to be more important as cotton parasites than *P. brachyurus* and *C. ornatum*.

Rainfall appeared to have little effect on nematode population changes, except possibly *H. tylenchiformis*, principally a migratory ectoparasite.—Agricultural Research Service, United States Department of Agriculture, and Departments of Botany and Plant Pathology, and Agronomy and Soils, Auburn University, Agricultural Experiment Station, Auburn, Alabama.

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LYSIS OF MYCELIUM OF PLANT-PATHOGENIC FUNGI BY NATURAL SOIL

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SUMMARY

Mycelium of most of 20 plant pathogenic fungi was virtually completely destroyed when living or killed 4-day-old cultures on agar media were covered with field soil for 1–2 weeks. There was no significant difference in the average lysis indices for living mycelium on peptone agar or on Czapek's agar, but propylene-oxide-killed cultures on peptone agar were more completely destroyed than those on Czapek's agar. The average lysis indices for root-infecting fungi and non-root-infecting fungi did not differ.

There is increasing evidence that a natural toxicity to fungi exists in most, if not all, soils. This toxicity is manifested by the nonspecific inhibition of germination of fungus spores (3, 4, 5, 8) and the lysis of fungal germ tubes and mycelium (5, 6). The mycolytic action of natural soil has been shown on mycelium of several plant pathogens growing in agar media (5). Sterilized soil inoculated with certain strains of *Streptomyces* spp. also lysed mycelium of fungi in agar (5). Since the lysis of mycelium by soil may affect the survival of plant pathogens in soil (7), the effect of natural soil on mycelium of 8 root-infecting and 12 non-root-infecting fungi was studied.

Materials and methods.—The root-infecting fungi used were *Aphanomyces euteiches* Drechs., *Fusarium solani* (Mart.) Appel & Wr. f. *pisi* (F. R. Jones)

Table 1.—Lysis of mycelium of root-infecting and non-root-infecting plant-pathogenic fungi by soil placed on living or killed 4-day-old cultures in peptone or Czapek's agar for 14 days.

Fungus	Lysis index [a]			
	Living mycelium		Killed mycelium [b]	
	Peptone agar	Czapek's agar	Peptone agar	Czapek's agar
Alternaria solani	0.5	2.8	0.0	2.5
Aphanomyces euteiches [c]	0.6	0.8	0.0	0.5
Ascochyta pisi	1.2	1.4	0.0	2.0
Botrytis cinerea	0.5	0.0	0.0	1.0
Ceratocystis fagacearum	0.0	0.0	0.0	0.0
Cladosporium cucumerinum	2.3	2.3	2.0	2.5
Colletotrichum circinans	1.5	1.8	1.0	2.0
Colletotrichum phomoides	2.0	1.7	0.5	2.5
Colletotrichum pisi	0.0	0.0	0.0	0.0
Fusarium solani f. *pisi* [c]	1.3	1.7	0.5	2.5
Gibberella zeae [c]	1.8	1.8	0.0	0.5
Glomerella cingulata	0.0	0.7	0.0	1.0
Helminthosporium sativum [c]	0.2	2.8	0.0	2.5
Monilinia fructicola	0.0	0.3	0.0	1.0
Pythium aphanidermatum [c]	1.4	1.8	0.0	1.0
Pythium sp. [c]	0.0	0.0	0.3	0.8
Rhizoctonia solani [c]	2.0	2.5	3.5	3.0
Sclerotinia sclerotiorum	0.0	0.0	0.5	2.0
Stemphylium sarcinaeforme	0.5	0.8	0.0	2.0
Verticillium albo-atrum [c]	3.8	2.0	0.0	1.0
Average [d]	1.0	1.3	0.4	1.5

[a] 0 indicates no mycelium present; 1, 1–5% of mycelium present; 2, 6–50% of mycelium present; 3, 51–90% of mycelium present; 4, 91–100% of mycelium present. Each figure is the average of readings of 2 plates from 3–5 tests. Average lysis index for both root- and non-root-infecting fungi was 1.3; for cultures on peptone and Czapek's agar, respectively 0.7 and 1.4; for living and killed mycelium, respectively 1.1 and 1.0. LSD 5% for these averages was 0.4.

[b] Cultures killed by propylene oxide vapor.

[c] Root-infecting fungi.

[d] LSD 5% = 0.6.

Snyd. & Hans., *Gibberella zeae* (Schw.) Petch, *Helminthosporium sativum* Pam., King, and Bakke, *Pythium aphanidermatum* (Edson) Fitz., *Pythium* sp., *Rhizoctonia solani* Kühn, and *Verticillium albo-atrum* Reinke & Berth. Non-root-infecting fungi were *Alternaria solani* (Ell. & Mart.) L. R. Jones & Grout, *Ascochyta pisi* Lib., *Botrytis cinerea* Fr., *Ceratocystis fagacearum* (Bretz) Hunt, *Cladosporium cucumerinum* Ell. & Arthur, *Colletotrichum circinans* (Berk.) Vogl., *Colletotrichum phomoides* (Sacc.) Chester, *Colletotrichum pisi* Pat., *Glomerella cingulata* (Ston.) Spauld. & Schrenk, *Monilinia fructicola* (Wint.) Honey, *Sclerotinia sclerotiorum* (Lib.) d By., and *Stemphylium sarcinaeforme* (Cav.) Wiltshire.

Procedure for assaying lysis of fungi by soil was described previously (5). Test fungi were cultured on peptone agar (pH 6.9) and Czapek's agar (pH 6.2). Cooled media in Petri dishes were inoculated with spore suspensions except for *A. euteiches*, the 2 Pythium species, and *R. solani*, which were seeded by mycelial transfer. An unsterilized mixture of 1 part Conover loam and 1 part muck was applied to 4-day-old living or propylene-oxide-killed cultures. Lysis data were taken 7 and 14 days later by microscopically comparing the amount of mycelium in cultures covered with steam-sterilized soil and cultures without soil. The rating system that Brian and Hemming (2) used for scoring spore germination of *Botrytis allii* Munn. was adapted to the estimation of lysis. Ratings were 0–4, with 0 indicating no mycelium present; 1, 1–5% of mycelium present; 2, 6–50% of mycelium present; 3, 51–90% of mycelium present; 4, 91–100% of mycelium present. Tests were repeated 3–5 times, with little variation from test to test.

Results.—With few exceptions, soil caused the disappearance of most of the mycelium of the cultures tested within 1–2 weeks (Table 1). Destruction of many cultures was virtually complete on either medium, whether cultures were living or killed. Living mycelium of approximately half the species had lysis indices of less than 1.0 on both media, indicating that scarcely any strands could be detected. The agar was no barrier to the lytic action since mycelium at the bottom as well as at the top of the agar layer was lysed. Mycelium of some fungi was more resistant, but few lysis indices were greater than 2.0. It is not known whether the remaining hyphae were viable. Conidia of *C. circinans*, *F. solani* f. *pisi*, *G. cingulata*, and *V. albo-atrum* from lysed cultures on peptone agar were previously shown to be viable (5). With the exception of a few weak hyphae, agar was never invaded by mycelium originating from the soil.

That the lytic effect of soil on mycelium in agar cultures was a genuine one and would occur in the absence of agar was shown by experiments with mycelia grown in liquid media. Washed mycelial mats from 7-day-old cultures of *F. solani* f. *pisi*, *G. cingulata*, and *V. albo-atrum* grown in peptone or Czapek's broth were placed on natural soil in Petri dishes. Both living and autoclaved mycelia of all treatments were

completely destroyed in 1 week or less. Mycelia were not affected by autoclaved soil.

The average lysis index for living cultures on peptone agar (1.0) was significantly larger than that for killed cultures (0.4), but when both media were considered together average lysis indices for living (1.1) and killed (1.0) cultures did not differ statistically. The average lysis index of cultures on peptone agar (0.7) was significantly lower than that for Czapek's agar (1.4), because of the significantly lower index for killed cultures on peptone agar. These differences, though statistically significant, were small, and are of questionable biological importance. Average lysis indices for root-infecting and non-root-infecting fungi were the same (1.3).

The lysis patterns for living and killed mycelium on the 2 media were not consistent from fungus to fungus. Living and killed mycelia of *G. cingulata* and *C. pisi*, for example, lysed readily on both media. The dark mycelium produced predominantly by *H. sativum* and *A. solani* on Czapek's agar was more resistant to lysis than the hyaline mycelium produced on peptone agar. Living mycelium of *Stemphylium sarcinaeforme* and *Sclerotinia sclerotiorum* lysed readily on peptone agar, but killed mycelium showed some resistance to lysis when grown on Czapek's agar. *V. albo-atrum* was resistant to lysis in the living condition on both media, but was readily lysed when killed. Some hyphae of *R. solani* and *C. cucumerinum* remained on both media, both in the living and killed condition. Other fungi were less distinctive in their lysis patterns. Direct parasitism of fungal hyphae by other microorganisms was never observed.

Discussion.—These results provide further evidence for the nonspecific lytic action of natural soil on fungus mycelium (5), and suggest that the important survival organs of plant pathogens in soil are resistant spores or sclerotia rather than vegetative mycelium. With the exception of *H. sativum*, all of the root-infecting fungi used and *S. sclerotiorum* produce resistant spores or sclerotia, and appear to survive in soil for long periods in the absence of a host (9). Lysis of mycelium by soil seems to be one aspect of a homeostatic mechanism acting to resist colonization of soil by fungi (7). Mycolysis by soil would seem to be most destructive to those organisms that do not produce resistant structures, and may explain the effectiveness of rotations in controlling such fungi.

The report by Boosalis and Scharen (1) that oospores but not mycelium of *A. euteiches* persisted in decayed pea residue in soil, and that viable mycelium as well as sclerotia of *R. solani* persisted in sugar beet residue in soil, supports the present findings. Mycelium of *A. euteiches* was readily destroyed by soil, whereas mycelium of *R. solani* did not disappear completely.

The lysogenic factors in soil are unknown, although the effect can be reproduced by sterilized soils inoculated with certain isolates of Streptomyces (5). Lysis by soil may possibly be an autolytic phenomenon triggered b antibiotics or other toxins or caused by the presence of cell-wall-decomposing enzymes. Both suggestions are speculative. Lysis of killed mycelium, however, cannot be due to autolysis, and must be due to the action of cell-wall-decomposing enzymes, presumably of microbial origin. The reason for the various lysis patterns for living and killed cultures on the 2 media cannot be explained until the lytic factors involved are discovered.—Department of Botany and Plant Pathology, Michigan State University, East Lansing.

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EFFECT OF ALCOHOLS ON SPORES OF SCLEROTINIA FRUCTICOLA AND OTHER PEACH FRUIT-ROTTING FUNGI IN CALIFORNIA

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SUMMARY

The order of toxicity of alcohols to spores of *Sclerotinia fructicola*, *Rhizopus stolonifer*, and *Gilbertella persicaria*, immersed for 1 min. at 20°C, was propanol > isopropanol > ethanol > methanol. *Sclerotinia* spores were most sensitive to alcohols tested; *Gilbertella* and *Rhizopus* sporangiospores behaved alike in ethanol and propanol. Five and 10 per cent alcohols, except propanol on *Sclerotinia* conidia, had no effect. Sixty per cent killed spores except for *Gilbertella* in isopropanol and *Rhizopus* in methanol. The most effective combinations of alcohols were propanol with ethanol or isopropanol. Fifty per cent ethanol can inactivate Sclerotinia conidia in 0.08 minute on peach fruit surfaces and in 2 minutes on the flesh. Sixty per cent ethanol killed *Gilbertella* or *Rhizopus* sporangiospores on uninjured surfaces of peach fruits in 1 minute, but on injured surfaces, 70% ethanol for 40 minutes was needed to kill *Gilbertella* and 70% for 60 minutes to kill *Rhizopus*. If immediately rinsed in water, there was no phytotoxicity on fruits immersed 1 minute in 60% ethanol. This treatment also produced firmer fruits with more intense ground and blush color.

Introduction.—The principal postharvest peach (*Prunus persica* Batsch.) fruit-rotting organisms in California were found to be *Rhizopus stolonifer* (Fr.) Lind., *Gilbertella persicaria* (Eddy) Hesseltine (4) and *Sclerotinia fructicola* (Wint.) Rehm [*Monilinia fructicola* (Wint.) Honey]. Studies were made on the effects of alcohols on spores of the above fungi and on the possible use of alcohol in postharvest disease control. Effects of alcohol have been reported most extensively for bacteria (4, 8, 9, 11) and viruses (1, 2). On fungi, toxic effects of alcohol have been reported on *S. fructicola* (10), *Penicillium expansum* Link. emend Thom (6), *Phytophthora colocasiae* Rac (12), and *Ustilago tritici* (Pers.) Rostr. (3).

Materials and methods.—*S. fructicola*, *R. stolonifer*, and *G. persicaria* isolates from peach were grown in Petri plates on 2% potato-dextrose agar (PDA). Spores were harvested by adding distilled water to release them and passing the suspension through 6 layers of cheesecloth. Methanol, ethanol, propanol, and isopropanol of reagent grade were diluted with distilled water to the required concentrations. Treatment of spores with alcohol consisted of: 1) floating, 2) immersion, 3) dipping of contaminated uninjured fruits, and 4) dipping of contaminated cm^3 sections of fruit (simulate injured fruits). Combinations were made by mixing equal volumes of the two alcohols. In immersion tests 4 ml of spore suspension (approximately 375,000 *Sclerotinia* and 1,125,000 *Gilbertella* and *Rhizopus* spores) were added to make the final volume of 10 ml at the desired alcohol concentration. After a one-minute immersion of conidia in alcohol, 2 drops (approximately 0.04 ml) of the alcohol spore suspension were transferred to 20 ml of distilled water (spores in 0.2% alcohol germinated and grew normally), and from this suspension 10 separate drops were transferred to PDA in Petri plates to determine kill. Peach sections with spores were also transferred to large volumes of water after treatment to reduce the concentration of spore germination and mycelial growth. In some instances the first macroscopic growth of the fungi was delayed 24–48 hours. Kill of spores was based on lack of growth in a 72-hour incubation period at 20°C. Field trials were made with 95% ethanol diluted in tap water.

Results and discussion.—*Alcohol effects on conidia.*—Table 1 shows the effects of various percentages of alcohols in water on spores of *S. fructicola*, *R. stolonifer*, and *G. persicaria* for 1-minute immersion periods.

The effectiveness of primary alcohols in spore kill in general increases with increased molecular weight of the alcohols. Isopropanol, a secondary alcohol, was not as effective as propanol and did not behave like methanol or ethanol. *Sclerotinia* conidia were most sensitive to kill. Propanol and isopropanol killed *Sclerotinia* and *Rhizopus* at lower concentrations than did methanol and ethanol. *Gilbertella* sporangiospores were more sensitive to propanol than to ethanol and

Table 1.—Effect of a 1-minute treatment of spores of *Sclerotinia fructicola* (S), *Rhizopus stolonifer* (R), and *Gilbertella persicaria* (G) in various alcohols of various concentrations in water.

	Alcohols											
	Methanol			Ethanol			Propanol			Isopropanol		
Conc. %	S	R	G	S	R	G	S	R	G	S	R	G
5	+[a]	+	+	+	+	+	+	+	+	+	+	+
10	+	+	+	+	+	+	(+)	+	+	+	+	+
15	+	+	+	+	+	+	0	(+)	(+)	0	+	+
20	+	(+)[b]	+	(+)	+	+	0	0	0	0	+	+
30	0	(+)	(+)	0	(+)	(+)	0	0	0	0	(+)	(+)
40	0	(+)	(+)	0	(+)	(+)	0	0	0	0	0	(+)
60	0	(+)	0	0	0	0	0	0	0	0	0	(+)

[a] + = growth on 10 transfers in 12 hours on PDA; 0 = no growth in 5 days.
[b] (+) = less mycelial growth than the control within 24 or 48 hours of transfer to PDA.

Table 2.—Effect of 1-minute treatments of spores of *Sclerotinia fructicola* (S), *Rhizopus stolonifer* (R), and *Gilbertella persicaria* (G) in combinations of various alcohols.

% of each alcohol	Methanol and ethanol			Methanol and propanol			Methanol and isopropanol			Ethanol and propanol			Ethanol and isopropanol			Propanol and isopropanol		
	S	R	G	S	R	G	S	R	G	S	R	G	S	R	G	S	R	G
5	+[a]	+	+	+	+	+	0	+	+	0	+	+	+	+	+	+	+	+
10	+	+	+	(*I*)	(+)	+	0	+	+	0	(+)	(+)	0	+	+	0	0	(+)
15	0	(+)[b]	+	0	0	0	0	(+)	(+)	0	0	0	0	0	(*I*)	0	0	0
20	0	(*I*)[c]	0	0	0	0	0	(+)	0	0	0	0	0	0	0	0	0	0

[a] + = growth on 10 transfers in 12 hours on PDA; 0 = no growth in 5 days.
[b] (+) = less mycelial growth than the control within 24 or 48 hours of transfer to PDA.
[c] (*I*) = mycelial growth present on a single transfer after 48 hours.

methanol. Isopropanol did not kill *Gilbertella* sporangiospores but delayed their mycelial growth in culture.

Effects of combination of alcohols on conidia.—One-minute immersion tests on each of the three pathogens were made with combinations of methanol, ethanol, propanol, and isopropanol (Table 2). Five per cent mixtures of methanol-isopropanol or ethanol-propanol produced the greatest toxicity to *Sclerotinia* conidia. In general, the most effective combinations were propanol with ethanol or isopropanol.

Sclerotinia was more sensitive than *Rhizopus* or *Gilbertella*; *Sclerotinia* conidia were killed by 5% of methanol-isopropanol or ethanol-propanol, 10% of ethanol-isopropanol or propanol-isopropanol, or 15% of methanol-ethanol or methanol-propanol.

Rhizopus was more sensitive than *Gilbertella* to these combinations. *Rhizopus* sporangiospores were killed by 10% propanol-isopropanol, or 15% ethanol-propanol, ethanol-isopropanol or methanol-propanol, but not by 20% methanol-ethanol or methanol-isopropanol.

All 20% and 15% mixtures of methanol and propanol, ethanol and propanol, or propanol and isopropanol killed *Gilbertella* sporangiospores.

Ethanol effects on Sclerotinia conidia.—Table 3 shows the ethanol concentration and exposure period required to kill conidia of *S. fructicola*. With exposure to alcohol for 0.08 minute, conidia floated on alcohol required higher concentrations for kill than conidia immersed in alcohol. This difference disappeared at longer exposure periods. At the lower alcohol concentrations, conidia on uninjured peach surfaces were killed with shorter exposure than conidia on injured surfaces.

Table 3.—Inactivation of *Sclerotinia fructicola* conidia with ethanol in relation to exposure period and concentrations.

Exposure (min.)	Conidia exposed in the following manner to ethanol							
	Floated		Immersed		On surface of peach fruit		On flesh of peach fruit	
	No[a]	Yes[a]	No	Yes	No	Yes	No	Yes
0.08	20[b]	30	30	40	40	50	60	
0.25	20	30	20	30	40	50	60	
0.50	20	30	20	30	40	50	60	
1.00	20	30	20	30		40	60	
1.00							70	
1.00							80	
2.00	20	30	20	30		40	40	50
15.00		10		10		40		40

[a] No = growth of fungus within 72 hours on PDA; Yes = spores killed, growth not present in 72 hours on PDA.
[b] Per cent ethanol.

Conidia on uninjured fruit surfaces were killed by 1 minute in 40% or 0.08 minute in 50% alcohol. Conidia on injured fruits required 15 minutes in 40% or 2 minutes in 50%. The difficulty of conidial kill on the injured fruit is probably related to the precipitation of fruit pectins, which prevents the alcohol from contacting the conidia.

Ethanol effects on Rhizopus and Gilbertella sporangiospores on fruits.—Ethanol treatments on uninjured fruits were less effective on *Rhizopus* and *Gilbertella* sporangiospores than on *Sclerotinia* conidia. Eighty-five per cent of the isolations from *Rhizopus*-contaminated uninjured fruits failed to produce mycelia after 0.08 minute treatment in 60% ethanol, whereas *Gilbertella* grew from each isolation; a 1-minute treatment, however, killed both organisms. A 70% concentration of ethanol took 45 minutes to kill *Rhizopus* sporangiospores on injured fruits, and 60 minutes to kill *Gilbertella* sporangiospores.

Phytotoxicity.—Boxes of Fay and Regular Elberta peaches dipped for 1 minute in 40% ethanol and not rinsed in water produced numerous brown superficial lesions in streaks. Kirkman Gem peaches dipped 10 seconds in 60% ethanol and immediately rinsed in tap water showed no injury, nor did Late Champion peaches dipped 2 seconds in 60% ethanol and immediately rinsed in distilled water.

Firmer fruits from ethanol dips.—Ethanol-dipped fruits were found to be firmer than the control fruits. Rio Oso Gem peaches were dipped 1 minute in 50% ethanol and not rinsed. After 10 days of storage, they withstood 0.69 lb. of pressure with a $\frac{5}{16}$-in. Magness-Taylor pressure tester (7), whereas untreated peaches could withstand only 0.30 lb. pressure.

Color of fruit improved with ethanol dips.—Immediate improvement of fruit color was noticed. The higher intensity of blush and ground color is mostly attributed to the cleansing of the fruit surface and removal of trichomes. Dodecyl benzene sulfonate, used in water

of the same surface tension as 60% ethanol, also improved appearance.—Department of Plant Pathology, University of California, Davis.

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SOIL WASHING IMPROVES THE VALUE OF THE SOIL DILUTION AND THE PLATE COUNT METHOD OF ESTIMATING POPULATIONS OF SOIL FUNGI

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SUMMARY

Soil washing which removes excessive numbers of detached spores of *Penicillium* and *Aspergillus* species improves the reliability of the soil dilution and plate count method for estimation of soil fungi. Number of genera of fungi and percentages of soil pathogens and other soil fungi were greater in plates of washed soils than in the unwashed soils. The number of *Fusarium* species of washed soils was in better agreement with root and bulb rot records of these fields.

Introduction.—The soil dilution and plate count method for the estimation of soil fungi has certain inherent weaknesses. There has been a tendency to assume that, once mixed in water, the soil and fungus particles remain in suspension. To make the dilution technique work most accurately, the fungus "growth units" should remain in a solution or suspension. The rapid settling of the particles in solution and the differential settling of the heavier, larger particles, if not corrected, makes soil dilution and plate counts of relatively less value for estimating the census of soil fungi.

Certain genera of fungi such as *Penicillium* and *Aspergillus* produce more growth units per unit of soil mycelium than do most of the plant parasitic fungi and most other nonparasitic fungi found in the soil. The soil dilution and plate count method measures only growth units. A viable spore produces a countable colony as readily as does a sclerotium or a large quantity of mycelium. Because of differences in fungus reproduction, spore production, and rates of growth, the soil dilution method favors the spore that produces a fast growing type of mycelium. The more abundant and rapidly growing *Penicillium* and *Aspergillus* species tend to overgrow, suppressing the growth of many other genera of fungi, and they are recorded in greater numbers than their actual mycelium concentration in the soil would warrant. The soil washing technique was developed to help correct for these obvious shortcomings in the soil dilution and plate count method for estimation of the numbers of fungi in the soil.

Materials and methods.—Soil samples were collected in the lower Snake River Valley from onion fields with a long history of cultivation and from newly cultivated desert soils, both including soils with good production records and soils infested with root- and bulb-rotting fungi. These soils were used for a comparative study on the recovery of soil pathogenic fungi and the estimation of fungus populations in the soil with the "soil washing" vs. standard soil dilution and plate count methods.

The dilution method used was essentially as described by Johnson et al (1). The fungi were cultured on a potato-dextrose agar, with 4% Bacto-oxgall added to restrict colony development and 100 ppm streptomycin to suppress bacterial development. The "soil washing" was done in a 500-ml Erlenmeyer flask with the equivalent of 1 g air-dry soil and about 200 ml of water for each wash. The soil was agitated thoroughly and allowed to settle with the flask on a 45° angle for 1 minute, and the water was then decanted. Dilutions were performed in the usual manner following the prescribed number of washes. The soil residue remaining following the washing was dispersed in water in a Waring blender. Soil dilution and plate counts were made to determine the fungi remaining in the residue. The percentages of the fungi found in the residue appear on some of the figures as "GR"—ground residue.

Results.—The settling that occurs while dilutions are made influences the counts of fungi (Fig. 1-A). The relative percentages of *Penicillium* and *Aspergillus* increased with a slight settling of heavier particles, whereas, the relative numbers of colonies of *Fusarium* spp. and all other fungi increased when the dilutions were made with continuous agitation. Being small and

light, the spores of *Penicillium* and *Aspergillus* species act more like suspended particles and remain suspended well in standard dilution plate-counting procedures. The other mycelium growth units, those attached to the soil particles, and the larger spores of certain genera of fungi behave more like falling bodies in the water. The errors were proportional to the time delay in making the dilutions.

Menzies (2) developed a cup-shaped "dipper" to avoid the obvious settling of soil particles. This settling can be seen readily in a glass pipette while dilutions are made. The settling of the heavier particles in the pipette likewise gives a greater number of *Fusarium* species and most other fungi in the first few ml taken from the pipette, and a higher number of *Penicillium* and *Aspergillus* species in the top part of the last few ml delivered from the pipette. Menzies' "dipper" corrects nicely for settling in pipetting and, because of its construction, aids in maintaining a constant agitation while samples are taken. The "soil washing" by removing the excess loose spores from the sample helps obtain a more realistic picture of the soil fungi. The plates from the washed soil revealed more of the soil-borne pathogens, more genera of soil fungi, and a larger number of fungi other than *Penicillium* and *Aspergillus*. Some plant pathogens and other genera of fungi present in the soil were seldom seen in the unwashed soil plates.

In tests using sterile soil mixed with spores of these fungi, it was evident that most of the loose spores were removed (90–99%) by the 4th wash (Fig. 1-B). Likewise, the early washings of soil were the most effective in removing spores and reducing the numbers of colonies of *Penicillium* and *Aspergillus* (Fig. 1-B, 1-C). The growth units of bacteria and actinomycetes were likewise removed more rapidly than the growth units of the soil fungi.

The number of colonies counted following washing when compared with unwashed soils shows a steady decline in the percentage of *Pencillium* and *Aspergillus* species and an increase with each washing in the percentage of all other genera of fungi plated out (Fig. 1-C). The percentage of colonies of the genus *Fusarium* increased from 2.0% in the unwashed to 35.8% at the 32nd washing. The numbers of genera of fungi identified were increased with the decrease in numbers of *Penicillium* and *Aspergillus* species (Fig. 1-C). The percentage of all other fungi increased from 39% of the total plate count of fungi in the unwashed soils to 66% of the fungi in the soils washed 32 times.

The washed soil was dispersed in a Waring blender and diluted, and plate counts were made. In the dispersed washed soil there was an increase in the numbers of total fungus growth units plated when compared to the plate counts at 32 washings. The amount of this increase of "growth units" in total numbers due to dispersal in the Waring blender varied with the type of soil washed. The total growth units in the washed dispersed soil were 60–90% of the original unwashed soil. Similar increases were observed in the numbers of bacteria and actinomycetes in this washed dispersed soil. The percentage of *Penicillium* and

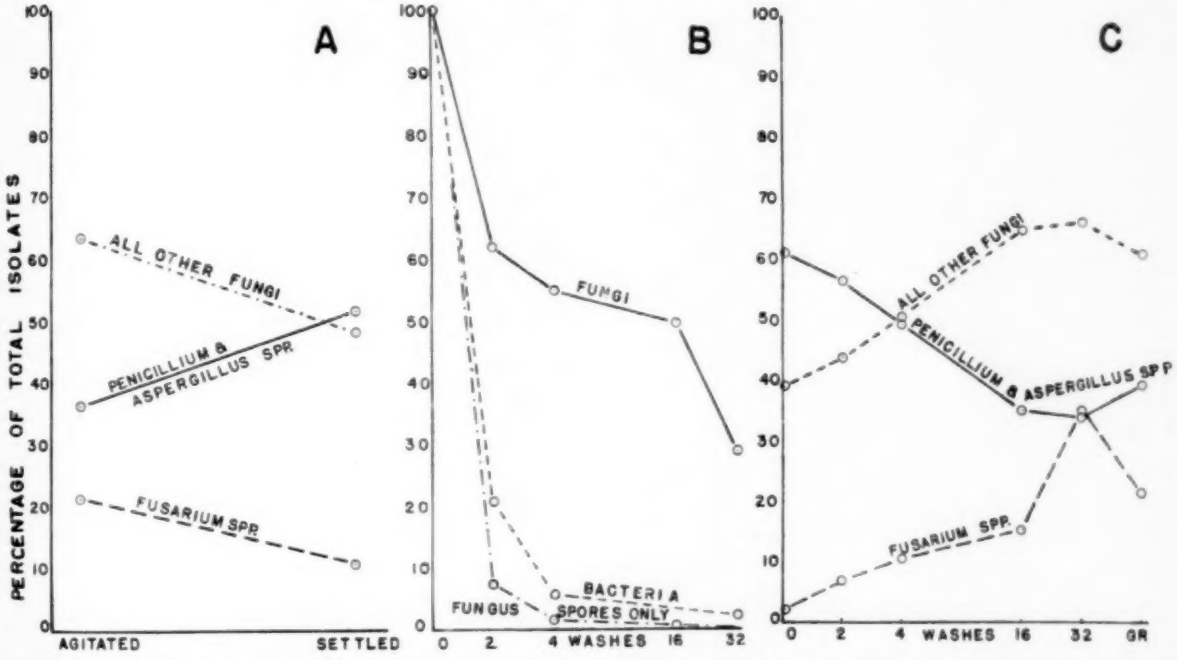

Fig. 1.—**A)** Effect of settling during sampling on the percentage of various fungus groups as determined by the soil dilution and plate count method. **B)** Soil washing reduces the total numbers of soil organisms. It reduces the percentage of loose fungus spores and bacteria at a much faster rate than it does the total growth units of all soil fungi. **C)** Soil washing changes the relative percentage of groups of soil organisms as determined by the soil dilution and plate count method. Grinding the washed soil residue (GR) increases total numbers of growth units and changes the direction of the curves.

Table 1.—Percentage of soil fungi on plates from onion soils in Idaho

Field No.	Soil not washed			Washed soil		
	Penicillium & *Aspergillus* spp.	All other fungi	Fusarium spp.	*Penicillium* & *Aspergillus* spp.	All other fungi	Fusarium spp.
1	13.9	86.1	33.0	4.2	95.8	63.0
2	52.8	47.2	10.6	23.0	77.0	21.1
3	68.3	31.7	10.6	49.2	50.8	20.3
4	57.8	42.2	6.3	30.1	69.9	29.1
5	32.2	67.8	8.6	9.8	90.2	29.3
6	67.0	33.0	11.6	43.0	57.0	24.3
7	78.8	21.2	4.8	46.2	53.8	15.0
8	55.2	44.8	11.4	20.8	79.2	25.7
10	60.0	40.0	7.8	10.1	89.9	47.8
11	78.9	21.1	6.4	42.9	57.1	28.6
12	45.0	55.0	21.7	15.4	84.6	38.5
13	45.5	54.5	3.7	29.0	71.0	40.7
14	11.1	87.9	17.2	5.0	95.0	67.2

Aspergillus colonies increased, as is shown by the upswing at the end of the curve (Fig. 1-C). There was a corresponding decrease of all other fungi and the *Fusarium* that were plated out. The net effect of soil washing and dispersing of the remainder usually resulted in a more varied population of genera of fungi and fewer units of the *Penicillium* and *Aspergillus* species, but the differences were not as marked as those following the washings.

The use of washed soil in the "soil-plate method for isolating of fungi from the soil" as suggested by Warcup (3) proved much superior to the unwashed soil. The washed soil residue produced a lesser percentage of the spore producers, a greater percentage of all other types of fungi, and a wider range of different genera of fungi than the untreated soil. For these reasons it was felt that this would be a worthwhile modification to the soil-plate method.

As a further evaluation of soil washing, samples from 13 selected onion fields were compared, washed and unwashed (Table 1, Fig. 2-A,B,C). These soils were selected to afford a wide variation in fungus population and included newly cultivated desert soils as well as ones from the older cultivated areas, some of which were poorly managed while others had been under good rotation programs for disease control.

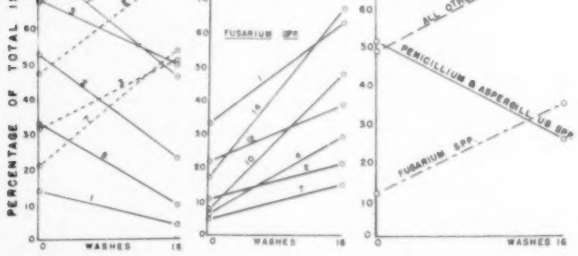

Fig. 2.—The relative percentages of soil fungi in 13 onion soils varied considerably. Soil washing **A)** reduced the *Penicillium* and *Aspergillus* species and increased the percentages of the other fungi, and **B)** decreased that of *Fusarium* species. The line numbers refer to field numbers (Table 1). **C)** Average percentage change for the 13 soils used in these experiments.

Variation was considerable between these field soils in content of the various organisms (Fig. 2-A,B,C). Agreement between the amount of *Fusarium* root rot and basal rot known to occur in these fields and the number of *Fusarium* species plated out was better in the washed series than in the unwashed. Fields 1, 10, 12, 13, and 14 were all known to have a long history of onion culture and a high incidence of Fusarium root and bulb rot problems. In the unwashed series, fields 10 and 13 were comparatively low in the numbers of *Fusarium* spp. plated out, whereas fields 1, 12, and 14 were comparatively high (Table 1, Fig. 2-B). In the washed series all of these fields were higher than the rest of the soils in the numbers of *Fusarium* spp. plated.

Fields 2, 3, 4, and 5 were newly cultivated desert soils, and fields 6, 7, and 8 were cultivated a longer time but with good rotations. In all of these, the number of *Fusarium* spp. plated out in the washed soils was comparatively low. In the unwashed soils, 2, 3, and 6 might be considered as high or above average in numbers of *Fusarium* spp. (Fig. 4-B), but the spread between fields was small. The relationship between root rot occurring in the field and the numbers of *Fusarium* species plated from these soils seems more closely related in the washed series than in the unwashed.—Department of Plant Pathology, University of Idaho Agricultural Experiment Station, Moscow.

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BIOASSAY OF THE SYSTEMIC ACTIVITY OF CYCLOHEXIMIDE SEMICARBAZONE IN CUCUMBER PLANTS

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SUMMARY

Bioassay with a cucumber extract (from antibiotic-treated plants) supplemented with Bacto-agar and dextrose was used for study of the systemic activity of cycloheximide semicarbazone in cucumber plants. *Pythium ultimum*, extremely sensitive to low concentrations of the antibiotic *in vitro*, was selected from several fungi as most suitable as a biological indicator. The bioassay provides direct evidence that cycloheximide semicarbazone enters and is found in different concentrations in various parts of treated cucumber plants. Whether application was by root or by leaf immersion, concentration was highest in the stem and leaves, next-highest in the roots, and lowest in the hypocotyls. When cycloheximide semicarbazone was applied to cucumber roots, concentration of the chemical in the plants increased over time. In addition, accumulation in the stem and leaves was to a level above that provided to the roots. Stem and leaf extracts from cucumber plants had a greater inhibitory effect on the activity of cycloheximide semicarbazone *in vitro* than extracts from either roots or hypocotyls. The last had the least effect.

Introduction.—The realization that antibiotics may be a potential force in plant chemotherapy has stimulated considerable interest in their systemic activity. Numerous reports have dealt with the absorption and translocation of various chemicals in plants (2, 3, 4, 7, 9, 10, 11, 13). For instance, the semicarbazone derivative of cycloheximide (Acti-dione), with which the present investigation is directly concerned, has been found to be systemically active in the treatment of certain plant diseases. Hacker and Vaughn (5) reported that this antibiotic induces pre-infection resistance to black stem rust (*Puccinia graminis* Pers. f. sp. *tritici* Eriks. & E. Henn.) in the spring wheat variety Onas. Hamilton, Sykolnik, and Sondheimer (6) found that this material was systemically active *in vivo* against the cherry leaf spot fungus, *Coccomyces hiemalis* Higgins. In those two studies the criterion for systemic activity was the absence of disease symptoms on plants treated with the antibiotic and inoculated with a pathogen.

The aim of the present investigation was to test a bioassay technique and obtain direct evidence on whether cycloheximide semicarbazone or its degradation product is absorbed and distributed to various parts of treated cucumber plants.

Materials and methods.—*Selection of the test organism.*—Several fungi isolated from diseased cucumbers were screened for a test organism suitable for use in the assay of cycloheximide semicarbazone. These fungi were *Fusarium oxysporum* Schlecht. emend. Snyd. & Hans., *Colletotrichum lagenarium* (Pass.) Ell. & Halst., *Rhizoctonia solani* Kuhn, and *Pythium ultimum* Trow. In a preliminary test of two slightly different methods of bioassay, one was effective and one was not. The paper disc-agar plate diffusion method was inadequate since no zones of inhibition developed in the "seeded" agar around paper discs impregnated with antibiotic in concentrations up to 1000 ppm. The other method, similar to the one finally adopted for determining antibiotic activity in cucumber plants, involved mixing known concentrations of the chemical with warm Difco potato-dextrose agar (PDA) just prior to autoclaving and pouring into Petri plates. After the agar had solidified, an agar disc 5 mm in diameter, containing a test organism, was placed in the center of each plate. After 24–30 hours colony diameters were measured to ascertain the inhibitive effect of the chemical on the various fungi treated. *Pythium ultimum* was selected as the best test organism for all assays because it grew rapidly and was very sensitive to the antibiotic, and the colonies had well defined margins.

General methods of bioassays.—Throughout this investigation the following procedure was adopted in the assay of cycloheximide semicarbazone in extracts from Green Prolific cucumber seedlings. When the primary and secondary leaves were fully developed, the plants were removed from flats of steamed soil and the soil was washed from their roots. They were then placed with their roots in tap water. The antibiotic was applied either to the primary leaves, by immersing them in the chemical in small glass vials, or to the liquid substrate in which the roots of the plants were immersed. Plants were treated for 18–29 hours under continuous illumination (400–500 ft-c) and at temperatures ranging from 70 to 78°F. On removal of the treated plants from the liquid substrate, the treated roots were thoroughly washed or the treated leaves were discarded.

Before the plants were assayed for antibiotic activity, they were wrapped in alminum foil, frozen, thawed, and macerated, and extracts were obtained with a Carver hydraulic press. The extract was then centrifuged and the supernatant was stored frozen. Untreated controls were prepared in the same manner. The assay medium consisted of the extract supplemented with 2% Difco Bacto-agar and 2% dextrose. The medium was autoclaved and cooled, then 15 ml portions were added to sterile Petri plates. After the medium had solidified, a 5-mm Difco PDA disc of uniform thickness, with the 2-day-old test organism (*Pythium ultimum*), was placed in the center of each assay plate. After 24–30 hours at room temperature the diameters of the colonies were recorded. Each treatment was replicated 5 times.

Table 1.—Inhibition of colony diameter of *Pythium ultimum* by various concentrations of cycloheximide semicarbazone when mixed with extracts from different parts of cucumber plants.

Test	Origin of extract	% inhibition at concentration (ppm) indicated					
		5	10	15	20	25	30
1	Hypocotyls	37	64	79	90	100	100
	Roots	18	38	47	58	71	82
	Leaves + stems	6	16	27	29	33	38
2	Hypocotyls	24	52	59	89	100	100
	Roots	23	35	57	64	88	98
	Leaves + stems	5	10	16	19	25	32

Standard mixtures of cycloheximide semicarbazone were prepared for comparison with unknown extracts from treated plants. Known quantities of the antibiotic were added to the extract of untreated controls. For each experiment the antibiotic standards and the extracts of unknown activity from treated plants were assayed at the same time and in the same manner. Percentage inhibition and dosage were respectively transformed to probits and logarithms. The data could then be plotted as a straight line, which, according to Bliss (1), should minimize experimental errors. The concentration of the antibiotic as estimated by the inhibition properties of extracts of treated plants was interpolated from such a straight line.

Results.—*Effect of cucumber extracts on the activity of cycloheximide semicarbazone.*—In two separate trials extracts from various parts of untreated cucumber plants (stems plus leaves, hypocotyls, and roots) were tested for their effects on the action of cycloheximide semicarbazone in inhibiting colony diameters of *Pythium ultimum*. Individual agar discs with the test organism were placed on assay media consisting of Bacto-agar, dextrose, and the extract (diluted 1:1 with distilled water) containing known concentrations of the chemical. Colony diameters were measured in 24–28 hours.

Extracts from various plant parts affect the activity of cycloheximide semicarbazone to different degrees (Table 1). The fungitoxic action of cycloheximide semicarbazone was reduced most by extracts from the stems and leaves and least by extracts from the hypocotyls.

Absorption of cycloheximide semicarbazone by cucumber plants.—Extracts taken from cucumber plants that had been growing in a 60-ppm solution of cycloheximide semicarbazone for various periods were assayed for antibiotic activity in the manner previously described.

With one exception, there was a progressive absorption of cycloheximide semicarbazone over a period of 26 hours (Table 2). During this time the concentration of the antibiotic in the plants increased gradually from zero to about 24 ppm.

Translocation of cycloheximide semicarbazone from roots.—Antibiotic concentrations were estimated in different parts of treated cucumber plants. After plants had grown for 20 hours in a 60-ppm solution of cycloheximide semicarbazone, they were harvested and divided into stems plus leaves, hypocotyls, and roots. Extracts then obtained were diluted 1:1 with distilled water, prepared for assay, and assayed as described.

The results are presented in Table 3. Cycloheximide semicarbazone diffuses into the roots and becomes distributed in different concentrations throughout the plant. Concentrations were greater in the stem and leaves than in roots or hypocotyls. The last contained the lowest concentration of the chemical. Accumulation in the stems and leaves reached a level above that supplied to the roots.

Translocation of cycloheximide semicarbazone from leaves.—In a preliminary experiment, primary leaves were placed individually in a solution containing 30 ppm of antibiotic in 10-ml glass vials. After immersion in the antibiotic solution for 29 hours, the primary leaves were discarded. The remainder of the plants were then assayed by the method described.

The bioassay showed that the antibiotic was absorbed and translocated out of the leaves. Colonies of the test organism in the untreated control were 74–76 mm in diameter, whereas that of the treatment were 45–47 mm.

In a similar experiment the primary leaves were immersed in 40 ppm of the antibiotic for 24 hours before assay of the antibiotic in the various plant parts. The primary leaves were discarded, and the remainder of the plants was divided into the following parts: stems plus leaves; roots; and hypocotyls. These parts were then assayed as before.

Antibiotic activity was detected in the stems, leaves and roots, but not the hypocotyls. The stems and leaves and the roots respectively contained an antifungal agent equivalent to about 16 ppm and 8 ppm of cycloheximide semicarbazone.

Discussion.—The investigation established that cycloheximide semicarbazone is absorbed and translocated within cucumber plants. This of course does not mean that the chemical will also be systemic in other plants or that other antibiotics will act the same in cucumber plants. It is well known that the absorption and translocation of antibiotics is dependent on the chemical and the plant treated.

From the results, which agree with those of other

Table 2.—Antibiotic activity in extracts from cucumber plants growing in a solution of cycloheximide semicarbazone (60 ppm) for various periods.

Period of immersion (hr)	Mean diameter (mm) of colonies[a]	Estimated concentration (ppm) of antibiotic
0	56	0
2	48	2
6	42	10
10	35	15
14	30	20
18	32	18
26	27	24

[a] Each value is an average of 5 replications. The LSD was 5.4 at the 5% level, 7.4 at the 1% level.

Table 3.—Antibiotic distribution in cucumber plants following the application of cycloheximide semicarbazone (60 ppm) to roots.

Parts assayed	% inhibition	Estimated antibiotic concentration (ppm)
Stems + leaves	40	64
Roots	31	16
Hypocotyls	20	7

workers, it is apparent that an antibiotic may be distributed in different concentrations to various parts of treated plants. According to Sander and Allison (12), 2-pyridinethiol-1-oxide applied to the primary leaf of cucumber seedlings reached high concentrations in the roots and low concentration elsewhere. Gray (4) reported that streptothricin applied by leaf immersion to a primary leaf of a bean plant, was translocated in small amounts to the roots and in high amounts to the tops of the plant. In the present study, regardless of the method of applying cycloheximide semicarbazone, an antifungal agent was in highest concentration in the stems and leaves, next-highest concentration in the roots, and lowest concentration in the hypocotyls.

Antibiotic activity in the various extracts of plant parts may not be directly attributed to the translocated cycloheximide semicarbazone, since the activity may be caused by a different compound arising from a change in the antibiotic supplied. Lemin and Magee (8) studied the movement of cycloheximide acetate-2-C^{14} in tomato plants and suggested that the activity was due not to the acetate but to free cycloheximide released in the plant. Accordingly there is the possibility that a change to free cycloheximide also occurs with the semicarbazone derivative of cycloheximide in cucumber plants. It is also conceivable that this change, depending on the metabolic site, could occur at different rates, which would result in a variation of antibiotic activity within a plant.

The present investigation established that extracts taken from various parts of untreated cucumber plants had different effects on the activity of cycloheximide semicarbazone *in vitro*. The effect of the various extracts on the activity of the antibiotic *in vitro* was compared to the distribution of the antibiotic in the plants. Extracts from stems and leaves had the greatest inhibitory effect on antibiotic activity, although these parts from antibiotic-treated plants contained the highest concentration of the antibiotic. This could be explained by assuming that there is a greater inhibition of fungitoxic activity *in vitro* than *in vivo*. Perhaps cycloheximide semicarbazone is changed within the plants either to a substance with greater activity or to a substance that is inhibited to a lesser degree.—Department of Botany and Plant Pathology, The Ohio State University, Columbus.

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PURIFICATION OF TURNIP MOSAIC VIRUS

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SUMMARY

The turnip mosaic virus was purified from infected *Nicotiana* or *Brassica* spp. by homogenization of tissue in 0.5*M* potassium phosphate (pH 7.5) followed by clarification of the homogenate by treatment with n-butanol and centrifugation. Infectivity and virus yields were always greater when the tissue was homogenized in 0.5*M* buffer rather than in weaker buffers or in water. Differential centrifugation and selective precipitation of the virus by adjusting solutions to pH 5.3 were used to obtain a highly infectious product showing strong anisotropy of flow, a single symmetrical schlieren peak in an analytical ultracentrifuge, and somewhat flexuous rods about 680 mμ long and 18 mμ in diameter. Exposure of the purified virus to low concentrations of phosphate and other common buffer salts, except borate, caused irreversible aggregation and partial loss of infectivity.

Introduction.—Many aphid-borne plant viruses are present in the host plant in low concentration and are relatively unstable in crude extracts. These viruses generally lose infectivity in host homogenates after aging a few days or when diluted beyond 10^{-3} to 10^{-4}.

Table 1.—Effect of homogenization in different media on the infectivity and sedimentation of turnip mosaic virus from *Nicotiana glutinosa*.

Treatment [a]	Local lesions [b]	
	No centrifugation	Centrifugation [c]
1. Expressed sap	28	5
2. Homogenized in water	33	1
3. Homogenized in 0.1*M*, pH 7.5, phosphate	49	2
4. Homogenized in 0.5*M*, pH 7.5, phosphate	281	267

[a] 0.5*M*, pH 7.5, phosphate was added to treatments 1, 2, and 3 to a concentration equivalent to that in treatment 4 after centrifugation but before assay.
[b] Average lesions per 9 half-leaves in an incomplete block.
[c] Supernatant after centrifuging 20 min., 12,000 rpm, No. 40 rotor of Spinco model L ultracentrifuge.

In general, many of the techniques used in the purification of more stable plant viruses have not been successful with most aphid-transmitted viruses, presumably because of their more labile nature. Thus, few of these viruses have been isolated in sufficient quantity or states of purity for physical and chemical studies, although several have been partially purified and their morphology observed in the electron microscope.

The turnip mosaic virus has properties similar to many other nonpersistent aphid-borne viruses. The virus in expressed sap loses infectivity in 2–3 days at room temperature or when diluted beyond 10^{-3} (10). The virus particles are slender, flexuous rods that are characteristically found when preparations from infected tissue are observed in an electron microscope.

Several other aphid-transmitted viruses have been purified. Among these may be mentioned the alfalfa mosaic (1, 9) and cucumber mosaic viruses (12), and more recently the cauliflower mosaic virus (7). The present report describes a method for the purification of the turnip mosaic virus and some of the properties of the virus.

Materials and methods.—The cabbage A strain of the turnip mosaic virus was used. The virus was cultured in *Nicotiana glutinosa* L., *N. multivalvis* Lindl., *Brassica chinensis* L., or *Brassica perviridis* Bailey. Young plants were generally inoculated at the 3–4-leaf stage. Inoculations were made by rubbing carborundum-dusted leaves with a cheesecloth pad soaked with a buffered homogenate of infected tissue of *N. glutinosa*. Tissue was usually harvested when the plants were showing systemic symptoms, 2–3 weeks after inoculation. Plants inoculated 2 weeks or longer usually contained more virus than plants infected for shorter periods.

Virus infectivity assays after the various fractionations were made by mechanical inoculation to *Nicotiana tabacum* L. (varieties Havana 38 or Havana 425), which produce necrotic local lesions 3–5 days after inoculation. An incomplete block design was used for the infectivity assays. In some cases virus activity was followed by serological tests with an antiserum prepared by injecting rabbits with partially purified virus.

For tests in studying the effect of different buffers or buffer molarity and pH on the virus during homogenization of tissue, the leaf midribs were removed and the laminae cut into small pieces 2–5 mm square. These small pieces were then mixed well and uniform lots were weighed out for use. Small quantities of tissue were triturated with a mortar and pestle, or homogenized in 50 cc centrifuge tubes using a motor-driven homogenizer. Larger amounts of tissue were homogenized for 3–4 minutes in buffer or water in a Waring blender. Generally 1.2 ml of liquid were used per g of tissue in order that the homogenate might be sufficiently fluid for efficient maceration. Buffered or neutralized thioglycolic acid was usually added to the homogenization liquid to a 0.1% concentration. This reducing agent appeared to have a protective effect on infectivity during the homogenization process but had little effect in subsequent steps. A small amount of Dow-Corning antifoam A was added to reduce copious foaming in the blender. In experiments in which treatment involved different buffer salts, different molarities or pH values of the same buffer, or organic solvents, samples after treatment were dialyzed overnight against a common buffer (0.05*M*, pH 7.5 potassium phosphate) in an Oxford multiple dialyzer before assay.

Butanol was redistilled over copper before use. All treatments involving extracts containing butanol and the ultracentrifugations were carried out at temperatures of 2–4°C. A Spinco model L ultracentrifuge was used to sediment virus from the clarified extracts in the preparative experiments, and a Spinco model E was used for the analytical sedimentation studies.

Experimental results.—*Clarification of homogenates.*—Several methods used in the initial purification of other plant viruses to remove the host chloroplastic material and to give clear extracts were tested with expressed saps or weakly buffered homogenates of infected tissue. Freezing leaves before homogenization, mild heating of extracted saps, or acidification of homogenates to pH values sufficient to cause denaturation of the chloroplastic protein all gave preparations of low infectivity, though each produced well clarified supernatants after slow-speed centrifugation. Emulsifying extracted saps with a mixture of butanol and chloroform in the cold, followed by recovery of the aqueous phase after slow-speed centrifugation (11), did not yield any infectivity in the clear extract.

Centrifugation alone was also tested in attempts to clarify saps. Longer periods and higher speeds of centrifugation than ordinarily used as an initial treatment were found to remove host chloroplastic material fairly effectively. Centrifugation for 30 min. at 12,000 rpm in the No. 30 rotor of the Spinco ultracentrifuge removed the major part of the green material from expressed saps or dilute buffer homogenates. This treatment, however, removed infectivity almost completely from the supernatants. Loss of infectivity during this centrifugation was more marked with the solanaceous

Table 2.—Effect of homogenization in different media on the infectivity of turnip mosaic virus from *Brassica perviridis*.

Treatment [a]	Local lesions [b]	
	No centrifugation	Centrifugation [c]
1. Expressed sap	132	92
2. Homogenized in water	101	128
3. Homogenized in 0.1*M*, pH 7.5, phosphate	299	230
4. Homogenized in 5 vol. 0.1*M*, pH 7.5, phosphate	334	316
5. Homogenized in 0.5*M*, pH 7.5, phosphate	361	387

[a] 0.5*M*, pH 7.5, phosphate was added to treatments 1, 2, and 3 to a concentration equivalent to that in treatment 5 before centrifugation.

[b] Average lesions per 12 half-leaves in an incomplete block.

[c] Supernatant after centrifuging for 15 min., 15,000 rpm, No. 40 rotor of Spinco model L ultracentrifuge.

hosts than with the crucifers. When the green pellets were resuspended in dilute buffer and assayed, the greatest virus activity was found in this fraction. This behavior suggested that the virus was either adsorbed or bound to some host component that sedimented under these conditions, or that it was highly aggregated.

1) *Effect of buffer pH and molarity during homogenization.*—The pH values of the extracted saps of *N. glutinosa*, *N. multivalvis*, *Brassica chinensis*, and *B. perviridis* were respectively found to be 5.3–5.5, 5.5–5.7, 6.0–6.2, and 6.1–6.2. These low values, especially in the case of the solanaceous hosts, indicated that the virus may have been aggregating in the expressed saps and was thus removed during clarification.

Different molarities and pH values of potassium phosphate buffers were used for homogenizing tissue and tested for their effect on the loss of virus during slow-speed centrifugation. It was found that buffers of sufficient capacity to maintain the resulting homogenate near or somewhat above neutrality prevented the sedimentation of virus during clarification and also resulted in more highly infectious preparations (Table 1).

Infectivity was always greater when tissue was homogenized in buffer that tended to raise the final pH of the homogenate, and greatest when the final pH was near neutrality. About 1-1.2 ml of 0.5*M*, pH 7.5, potassium phosphate buffer or its equivalent was required per g of tissue to give a final pH above 7 with the hosts mentioned above. When various molarities of buffer were compared by homogenizing in the same buffer-to-tissue ratios, the 0.5*M* buffer, pH 7.5, was always superior to more dilute buffers (Table 1).

Expressed saps adjusted to pH 7.0–7.5 with dilute alkali were tested in attempts to avoid the diluting effect of homogenizing in buffer. The sap was expressed by grinding the tissue with a mortar and pestle or meat grinder, followed by squeezing the material through cheesecloth and adjusting the pH. Assays comparing this treatment, after addition of equivalent quantities of phosphate, with material homogenized in 0.5*M* buffer showed the latter to be greatly superior in infectivity. This result might have been due to a less efficient grinding than occurred in the blender, but the effect of grinding in water suggested that such was not the case. Material ground in water and subsequently brought to the same pH and phosphate concentration by dialysis or by addition of strong phosphate before dilution and assay, showed markedly less infectivity than material homogenized in strong buffer (Table 1, 2).

Results with phosphate buffers of greater ionic strength suggested that the effect may have been due to the higher salt concentration rather than to pH. Adjusting aliquots of a clarified 0.5*M*, pH 7.5, homogenate to various lower pH values, followed by centrifugation and dialysis of the various fractions against a common buffer before assay, showed that the higher resultant pH rather than the ionic strength was responsible for the greater infectivity and lack of sedimentation during slow-speed centrifugation (Table 3).

A number of other salts that buffer well near or somewhat above neutrality were tested as buffers during homogenization. The following were compared with 0.5*M*, pH 7.5, potassium phosphate by homogenizing uniform lots of infected tissue at the rate of 1 g of tissue per ml of buffer: 0.2*M* borate, pH 8.0; 0.25*M* glycine, pH 7.5; 0.12*M* Tris, pH 7.8; and 0.1*M* veronal, pH 8.0. The pH values of resulting homogenates were 7.0–7.6, but none of these buffers resulted in infectivity titers as high as those with phosphate.

2) *Treatment with butanol.*—Others (6) have found that n-butanol has a solubilizing effect on certain proteins. It was used initially to test its effect on preventing the sedimentation of turnip mosaic virus during slow-speed centrifugation and because Steere (11) had found that in combination with chloroform it was effective in removing extraneous protein from saps containing the tobacco ringspot virus. In the present study it was found that treatment of buffer homogenates with n-butanol alone was sufficient to precipitate the host chloroplastic material without inactivating the turnip mosaic virus.

Table 3.—Sedimentation of turnip mosaic virus in a clarified 0.5*M* buffer extract of *Brassica perviridis* after adjusting to various pH values.[a]

pH	Average no. lesions per 6 half-leaves [a]	
	Supernatant	Pellet [b]
7.3 (control)	417	35
6.0	145	26
5.5	1	108
5.0	1	73
4.5	0	51
4.0	0	1

[a] Centrifuged 15 min., 10,000 rpm, in No. 40 rotor of Spinco model L ultracentrifuge.

[b] Pellets resuspended by placing on a mechanical shaker for 2 hours in a volume of 0.05*M* phosphate buffer equal to that of the supernatant.

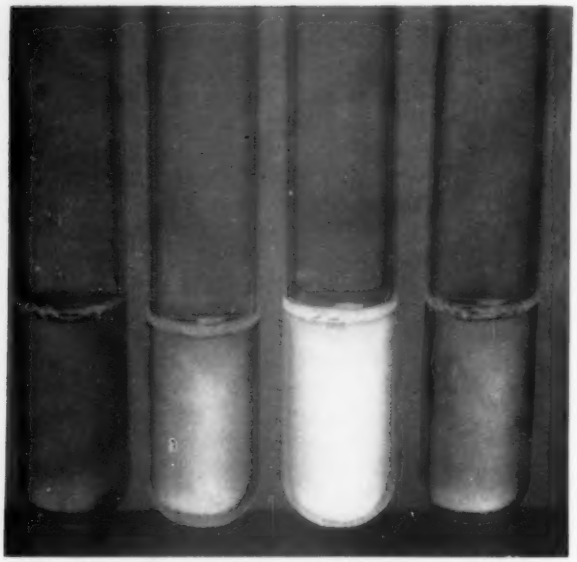

Fig. 1.—Aggregating effect of phosphate on the turnip mosaic virus. Left to right, tubes containing distilled water; virus in water; 0.02*M*, pH 7.3, phosphate; and 0.02*M*, pH 7.3, borate. The virus concentration is the same in each of the 3 tubes on the right.

The solubility of n-butanol in water is inversely related to temperature. It was found that a solution of about 10% butanol could be reached in buffered host homogenates at temperatures of 2–5°C. A concentration of 8–10% butanol gave efficient denaturation of the host chloroplastic material after incubating for 30 min. or longer. Centrifugation of treated homogenates for 15 min. at 5000 rpm in an angle rotor resulted in clear supernatants containing almost all of the infectivity.

Longer incubation with butanol appeared to result in less virus aggregation during subsequent treatment. In general, virus yields during subsequent steps were greater when 0.5*M* buffer homogenates were incubated overnight at 2–4°C with 8% butanol rather than 30–60 min. with 10% butanol, although the latter treatment did not decrease infectivity. The method that worked best was to cool the buffer homogenate to about 5°C after filtration through 2 thicknesses of cheesecloth. The homogenate was then placed on a magnetic stirrer and the butanol added dropwise to the constantly stirred homogenate from a small separatory funnel at a rate approximately equal to that at which the butanol went into solution. The homogenate was then incubated overnight at 2–4°C with stirring.

Ultracentrifugation and pH precipitation.—Following clarification the buffer extracts were centrifuged at high speed to separate the virus. Generally, clear gelatinous pellets resulted from one high-speed centrifugation. Various buffers were tested as resuspension fluids, but water was found superior to all and resulted in less aggregation and loss of virus during subsequent treatment. The pellets were usually covered with water and placed on a gently moving mechanical shaker for several hours for resuspension. If the virus, resuspended in water, at this stage was again given a cycle of differential centrifugation, the bulk of the material in the pellets remained insoluble in water after the second ultracentrifugation. Precipitation at acidic pH values was found to be a more convenient means of further purification of virus at this stage, and resulted in less tendency for aggregation during subsequent treatment.

Previous results with the insolubility of turnip mosaic virus at lower pH values suggested that acid precipitation might be useful in further purification. Tests showed that the virus could be precipitated quantitatively by adjusting concentrated aqueous solutions to pH 5.5–5.0. The formation of flocculent precipitates that could be easily removed by slow-speed centrifugation at these pH values was found to require small amounts of salt.

Behavior during acidification suggested the isoelectric point of the virus may be near pH 5.3. On several occasions water solutions of virus were carefully adjusted to various pH values from neutrality to pH 4.5 while turbidity readings were taken by determining the absorbance at 420 mμ. Maximum turbidity was at pH 5.3. On other occasions, however, preparations of the virus increased in turbidity as pH was lowered to 5.5–5.3 but did not become less turbid at lower pH values.

As used in the purification of the turnip mosaic virus, pH precipitation was found to be a convenient method to remove much host nucleoprotein, which sedimented from butanol-clarified supernatants during ultracentrifugation. After the first ultracentrifugation, virus was resuspended in water to a volume about 1/10th that of the original homogenate. This material was given a slow-speed centrifugation after the addition of pH 7.5 sodium borate buffer to a final concentration of 0.02*M*. Dilute HCl was added dropwise with rapid stirring to this solution to adjust the pH to 5.3. Solutions generally became markedly opalescent below pH 6, and a flocculent precipitate appeared near pH 5.5–5.3. When a flocculent precipitate did not appear at this pH after stirring for a few minutes, small quantities of solid NaCl were added until the precipitate

Table 4.—Aggregating effect of various salts on turnip mosaic virus.

Salt added[a]	Optical density at 420 mμ at various times after adding buffer salt[b]		
	10 min.	30 min.	24 hrs.
Water	—0.005	0.004	0.068
Ammonium sulfate	0.258	0.317	0.340
Citrate	0.00	0.00	0.050
EDTA[c]	0.100	0.150	0.160
Phosphate	0.008	0.006	0.040
Sodium chloride	0.050	0.062	0.229
Tris[d]	—0.012	—0.005	0.056

[a] All salts added to a final concentration of 0.02*M*, pH 7.3.

[b] Virus containing 0.02*M*, pH 7.3, borate used as control preparation (O.D. = 0.0).

[c] Ethylene diamine tetraacetic acid.

[d] Tris(hydroxymethyl) aminomethane.

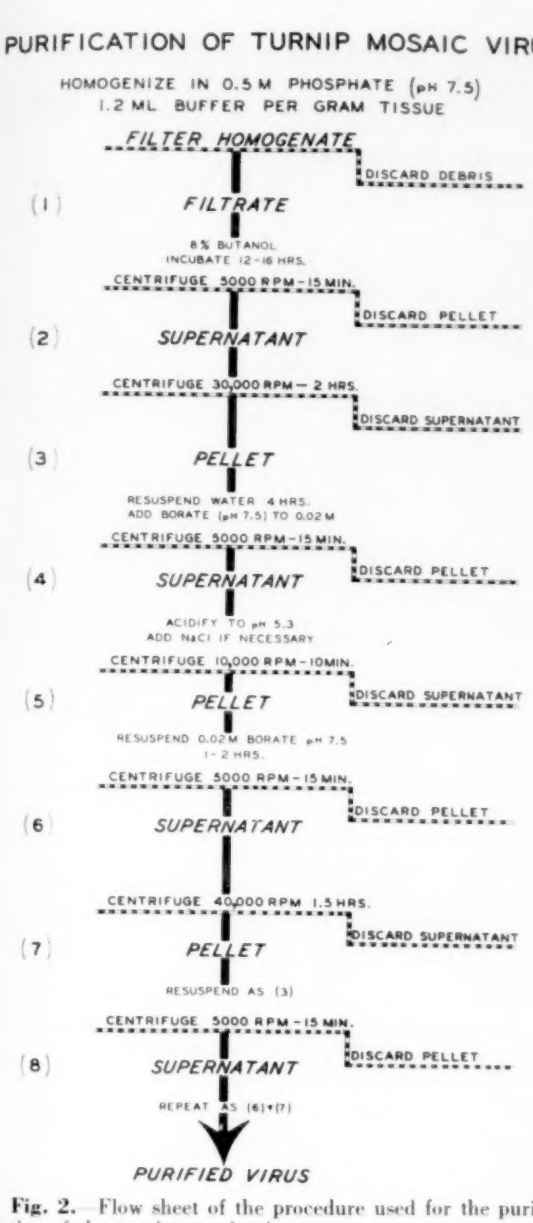

Fig. 2.—Flow sheet of the procedure used for the purification of the turnip mosaic virus.

formed. This precipitate was removed by centrifuging for a few minutes at 10,000 rpm and resuspended in 0.02*M* borate buffer of pH 7.5. Occasionally virus was not removed completely by the first treatment; however, the use of a small additional quantity of NaCl, followed by centrifugation, removed all the virus. Tests showed that the supernatant following acid precipitation was not infective and did not exhibit anisotropy of flow or possess any serological activity; however, examination of the supernatant in an ultraviolet spectrophotometer showed that considerable nucleoprotein was present.

Following acid precipitation, solutions were stirred for a few hours to effect complete resuspension of virus and given a slow-speed centrifugation to remove insoluble material. The virus generally showed more infectivity following this treatment (Table 5).

Aggregating effect of certain buffer salts.—As mentioned previously, it was observed that resuspension of pellets from ultracentrifugation in dilute phosphate, pH 7.5, resulted in less infectivity than resuspension in water. It was also observed that when partially purified preparations were diluted with phosphate for infectivity assays, precipitates appeared when the virus solution was pipetted into the phosphate. These precipitates were removed by slow-speed centrifugation and found to contain the infectivity. This apparent aggregating or precipitating effect was also found with other common buffer salts.

The aggregating effect of various salts was easily tested by adding the salt solution to a fairly concentrated aqueous solution of virus and observing the resultant increase in opalescence or turbidity (Fig. 1). In some cases, aggregation was not apparent immediately but developed after a few hours at room temperature and appeared to vary somewhat with the concentration and previous treatment of the virus preparation. Borate was the only buffer found to be suitable for use with partially purified turnip mosaic virus, for it was the only one tested that did not cause aggregation. Virus solutions containing borate remained clear for several days, in contrast to other salts tested. Table 4 shows the increase in turbidity of virus solutions containing other buffer salts after incubation for various periods at room temperature.

A flow sheet showing the purification procedure found to be most successful with the turnip mosaic virus is shown in Fig. 2. Infectivity titers at various steps during purification are shown in Table 5.

Analytical ultracentrifugation and electron microscopy.—Purified turnip mosaic virus in 0.2*M*, pH 7.3, borate showed a single schlieren peak during sedimentation in an analytical ultracentrifuge (Fig. 3-A), indicating that a single component was present. Some purified preparations showed small amounts of material that sedimented very rapidly unless the preparation was first centrifuged for a few minutes at 20,000 rpm. If the last cycle of differential centrifugation was omitted, virus preparations showed small amounts of more slowly sedimenting material.

Virus preparations to which phosphate was added

Table 5.—Infectivity of turnip mosaic virus from *Brassica perviridis* after various treatments during purification.

Treatment	Lesions per half leaf [a]
1. 0.5*M* buffer homogenate	193
2. Homogenate after addition 8% n-butanol	73
3. Homogenate with butanol after slow speed centrifugation	208
4. Resuspension after 1st ultracentrifugation	98
5. Resuspension after precipitation at pH 5.3	188
6. Resuspension after 2nd ultracentrifugation	119
7. Resuspension after 3rd ultracentrifugation	126

[a] Average number from 9 half-leaves in an incomplete block after dilution to a volume equivalent to the original homogenate.

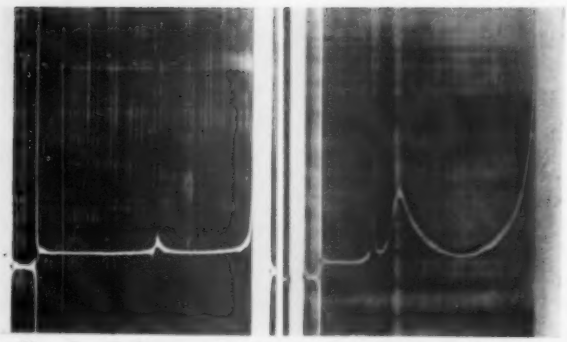

Fig. 3.—Sedimentation diagrams of turnip mosaic virus. **A)** Virus in 0.02*M*, pH 7.5, borate. **B)** Virus in 0.02*M*, pH 7.5, phosphate.

immediately before running in an analytical ultracentrifuge showed, in addition to large amounts of material that sedimented very rapidly, two schlieren peaks (Fig. 3-B). The slower migrating peak moved at about the same rate as virus in borate, and was probably nonaggregated virus. The larger, more rapidly moving peak was apparently aggregated virus, since this component was absent in similar preparations in borate.

The virus preparation whose sedimentation diagram is shown in Fig. 3-A, gave a dilution end-point on tobacco of 10^{-6}. Other similar preparations at about the same concentrations showed dilution end-points of 10^{-5} to 10^{-7}. This indicated that the single component observed during ultracentrifugation was the virus.

Electron microscopy of purified turnip mosaic virus showed numerous rods, and no other characteristic particle (Fig. 4). The small spherical particles frequently associated with host microsomal material were not present. The virus rods were about 680 mμ long and 18 mμ in diameter. Most of the virus rods from preparations containing borate were of fairly uniform length, though some longer and shorter particles were observed.

Serology.—An antiserum with a precipitin titer in excess of 1/512 was prepared by injecting rabbits with partially purified freeze-dried virus suspended in saline. The virus was insoluble after freeze-drying but retained some infectivity. The antiserum did not react in precipitin tests with the cauliflower mosaic, turnip yellow mosaic, or turnip crinkle virus.

Discussion.—The low pH of the extracted saps of certain plants is probably due to the mixing of acid vacuolar contents with the cytoplasm during the breaking of cells. The resultant low pH is sufficient to denaturate part of the cytoplasmic protein (13). A similar situation appears to apply in the case of some plant viruses. Partial loss of infectivity and insolubility of turnip mosaic virus occurs unless the pH is adequately controlled during homogenization. A similar loss of infectivity unless tissue is homogenized in buffer has been observed with alfalfa mosaic virus (5). Many host plants, e.g., members of the solanaceae and cruciferae as observed in the present study, have acid saps; others, such as certain legumes and cucurbits, have almost neutral saps. In cases where a given virus has a wide host range a choice of host may be available so as to avoid those with acid saps.

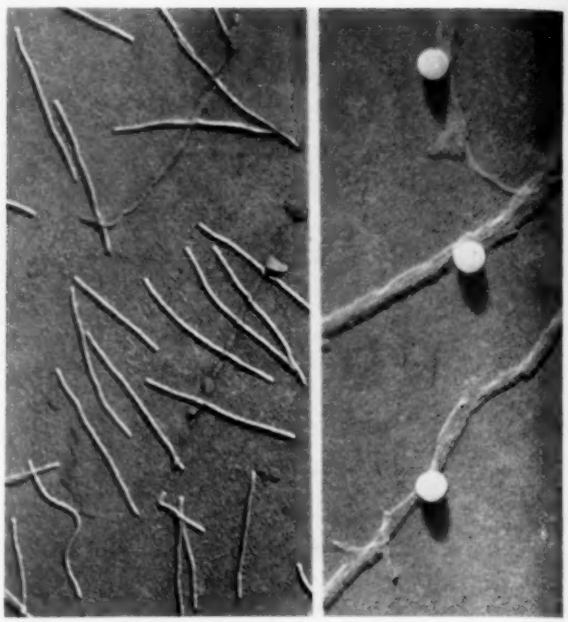

Fig. 4.—Electron micrographs of turnip mosaic virus. **A)** Nonaggregated virus. **B)** Aggregated virus. (Both × approximately 41,000). The large white spheres are polystyrene latex particles 1225 Å in diameter.

The aggregating effect of certain salts on the turnip mosaic virus has been observed with other viruses of similar morphology. Phosphate has been observed to induce aggregation and to inactivate potato virus X, particularly at elevated temperatures (2). Proteins and the materials in plant sap protected against this inactivation. Several other salts were also found to have similar inactivating effects, but not borate or veronal buffers. The latter buffer, but not borate, was found to cause aggregation of turnip mosaic. Methods have recently been described for the preparation of nonaggregated tobacco mosaic (4) and potato virus X (8). Neither method, however, was successful with the turnip mosaic virus.

Analytical ultracentrifugation and electron microscopy indicate that turnip mosaic as purified here is essentially pure, with characteristic particles associated with infectivity. The dimensions of the virus particles are not quite in agreement with those reported by others (3), but appear shorter and of greater diameter.—Department of Plant Pathology, University of Wisconsin, Madison.

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THE EFFECT OF AIR TEMPERATURE ON MULTIPLICATION OF TOBACCO MOSAIC VIRUS IN SUSCEPTIBLE AND RESISTANT PEPPER

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SUMMARY

The effect of air temperature of 16, 20, 24, and 28°C on multiplication of TMV in resistant and susceptible pepper was studied. Susceptible varieties, as represented by California Wonder, developed systemic mottle and chlorosis, and virus concentration initially increased as temperature increased. The reverse was obtained with later assays, virus concentrations being greater at 16° than at 28°. Resistant Yolo Wonder plants developed systemic chlorotic lesions at 16° but systemic necrosis at 28°. In all assays virus concentration increased with increased temperature. Virus was not recovered from the very tip leaves of plants at 16° and 20°. Resistant plants of P.I. Accession 163201 developed only necrotic lesions on inoculated leaves. These were rapidly lost by abscission. At no temperature and with no method of inoculation did systemic invasion occur. Delaying abscission of inoculated leaves by as much as 6 days by use of indoleacetic acid did not result in systemic invasion.

Introduction.—In 1934, Holmes (3) reported that a single dominant gene in certain pepper varieties (e.g. Tabasco) controlled the localization of tobacco mosaic virus (TMV) in inoculated leaves. In a later paper (4) he reported that three genes (*L*, l^1, and *l*) in an allelic series controlled all the reactions to TMV in the pepper varieties he studied. The gene *L* controls the local-lesion reaction, restricting the virus to inoculated leaves. This gene is completely dominant over l^1, which effects imperfect localization of the virus, and over *l*, which permits full systemic spread. Gene l^1 is partially dominant over gene *l*.

In 1952, Porter and Younkin (6) announced the development of Yolo Wonder pepper, a TMV-resistant variety derived from the cross California Wonder (susceptible) × Elephant Trunk (resistant). The reaction of Yolo Wonder to TMV was described as follows: "Yolo Wonder is resistant to tobacco mosaic virus because the virus does not readily move out of the inoculated leaves. If plants are artificially inoculated, relatively large lesions develop on inoculated leaves which drop soon after the necrotic spots appear, thus preventing virus movement in the plant."

The expression of resistance to viruses by plants has been shown in some cases to be markedly affected by the air temperature at which the plants are grown (7, 8). This paper reports a study of the effect of temperature on the multiplication of TMV in peppers of various genotypes and on plant reactions at various temperatures.

Materials and methods.—Seeds of 128 accessions of *Capsicum frutescens* L. were obtained from the Southern Regional Primary Plant Introduction Station and assayed for resistance to TMV. Accession no. 163201 was found to be heterozygous for the local-lesion gene. Some plants showed only necrotic lesions on inoculated leaves whereas others showed only a systemic mottle. Selfed progenies of plants of each genotype yielded only the two types and in ratios indicating that only the two genes *L* and *l* were present. This accession was selected for further studies, and plants of *ll* composition (herein referred to as 163201-S) were separated from those of *LL* (herein referred to as 163201-R) and *Ll* by the following method. The cotyledons or the first two true leaves of labeled plants were inoculated and excised immediately for incubation in metallic trays on 2% water agar. Within 2–3 days the leaves from plants containing the local-lesion gene could be easily distinguished from others by the conspicuous necrotic lesions (Fig. 1). This method was quick and gave absolute separation of the plants into resistant (163201-R, primary lesions only), and susceptible (163201-S, systemic symptoms).

The commercial varieties of *Capsicum annuum* L. used were obtained from the Associated Seed Growers, Inc. These were California Wonder (lot no. 57102), and Yolo Wonder (lot no. 37246).

Seeds were sown and plants were grown in quartz sand watered with a balanced nutrient solution. Inoculations were made by rubbing two lower carborundum-dusted leaves with a purified preparation of TMV.

The inhibitory action of pepper juice against viruses (5) was obviated by diluting infectious sap or by

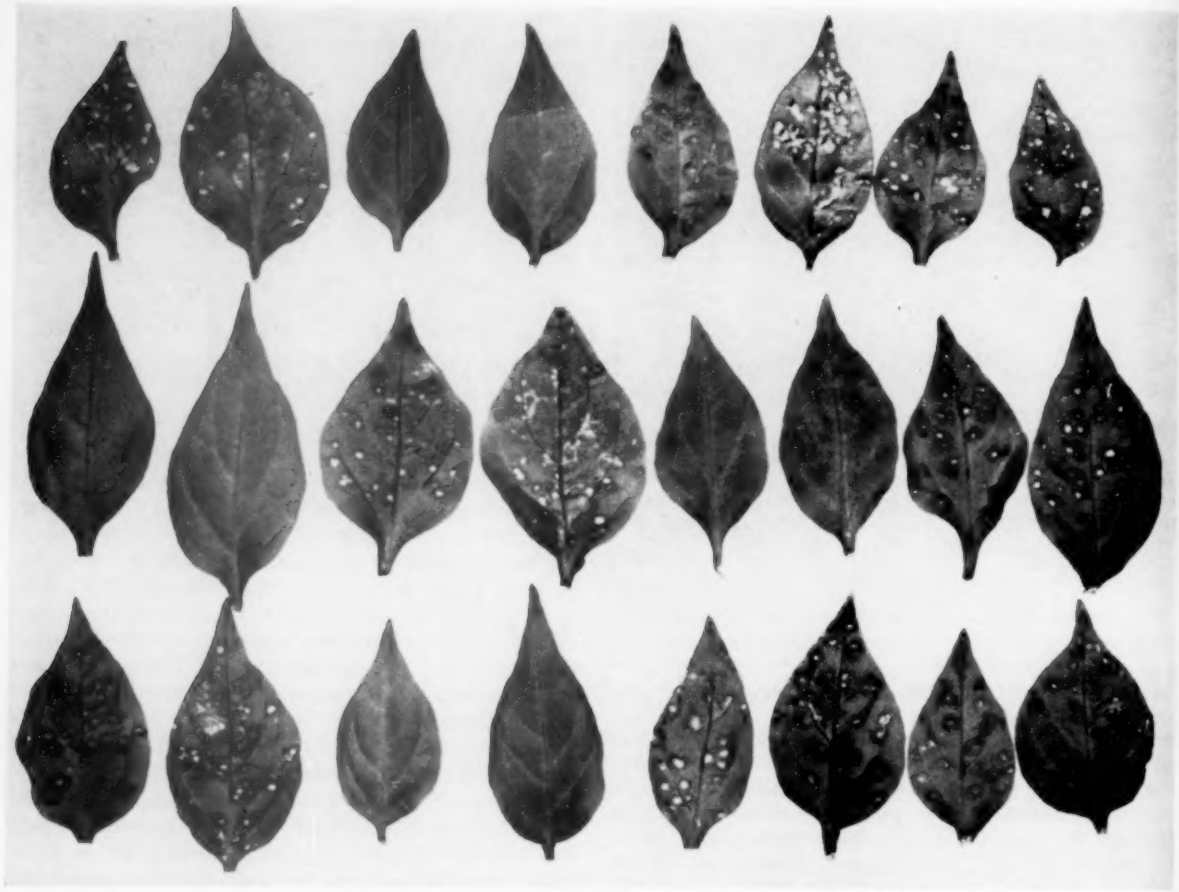

Fig. 1.—Symptoms on inoculated leaves excised and incubated on water agar. Each of 12 plants is represented by paired leaves. Note the separation into 2 distinct classes.

heat clarification (65°C for 15 minutes, followed by low-speed centrifugation). Thus, by diluting sap 1:2000 followed by heat clarification, the inhibitor potency was doubly neutralized (Table 1).

In practically all experiments, virus concentration was determined by both local-lesion and optical-density assays. In preparing inoculum for assay, sap samples were obtained from frozen tissue by trituration with a mortar and pestle for inoculated and tip leaves, and with a Waring blender for composite leaf samples. Distilled water was used as a diluent. Sap aliquots, in equivalent dilutions, were used as assay samples. For local-lesion assays heat-clarified sap extracts were used. Ultraviolet absorbency (O.D.) readings were made of preparations purified by two cycles of low-high-speed centrifugation following heat clarification. Temperature comparisons by local-lesion assay were made by the incomplete block design used by Pound (7), using plants of the F_1 hybrid of *Nicotiana glutinosa* × *N. tabacum*.

Results.—*Symptoms.*—California Wonder plants and susceptible plants of Accession 163201 did not exhibit the conspicuous mottle found in outside plantings, but rather a general chlorosis that resulted in a bleached appearance (Fig. 2). Symptoms were more severe in winter experiments, when light intensity and photoperiod were reduced, than in spring experiments. In the spring, symptoms on inoculated leaves were generally absent at 16 and 20° and abscission did not occur; at 24 and 28°, inoculated leaves developed chlorosis and abscised about 16–18 days after inoculation. Primary symptoms were never confined to discrete necrotic lesions.

Table 1.—Effect of dilution and heat clarification (65°C for 10 min.) on infectivity of sap from TMV-infected pepper plants.

Dilution	No. local lesions [a]	
	Untreated sap	Heat-clarified sap
0	101	[b]
1:10	133	[b]
1:100	226	[b]
1:1000	[b]	[b]
1:2000	310	460
1:4000	173	210

[a] The untreated and clarified saps were compared in half-leaf inoculations on *Nicotiana glutinosa*. Values are averages of 6 half-leaf replications.

[b] Lesions were too numerous for accurate count.

Fig. 2.—Systemic symptoms produced by TMV on Yolo Wonder (left) and California Wonder (right) pepper plants. Note blotchy necrotic lesions on Yolo Wonder and the general chlorosis on California Wonder.

Temperature affected both the rate of development and severity of systemic symptoms. Incubation periods increased with decrease in temperature. At 28° the characteristic syndrome was a prolonged and severe chlorosis that, in the winter, was followed by stem necrosis and death. At lower temperatures, especially in the spring, the chlorosis was less pronounced and less prolonged and tip leaves developed necrosis.

Yolo Wonder was used as a very tolerant variety but one in which systemic infection readily occurred. Distinct necrotic lesions developed on inoculated leaves at 16, 20, and 24°. At 28° the lesions were indistinct, and severe chlorosis and necrosis occurred within 4 days. In the spring, inoculated leaves at 16, 20, 24, and 28° abscised in about 14, 11, 6, and 5 days, respectively. In the winter, abscission was slightly earlier. Severity of systemic symptoms increased with increased temperature. At low temperatures, systemic symptoms consisted of large blotchy chlorotic lesions. At high temperatures the lesions were necrotic and leaves abscised progressively (Fig. 2, 3). Stem streak was also common (Fig. 3).

Resistant plants (163201-R) produced only necrotic lesions on inoculated leaves. At all temperatures, inoculated leaves abscised and the plants remained free of systemic infection. The time required for lesion appearance decreased with increase in temperature, and rate of leaf abscission increased.

Effect of temperature on virus multiplication.—In California Wonder and 163201-S plants, virus concentration in inoculated leaves at 6 days after inoculation increased with increase in temperature. In composite leaf samples and in tip-leaf samples of these two varieties, virus concentration patterns were somewhat similar (Table 2, 3) to the cyclic pattern in tobacco and tomato (1). Initially, virus concentration

Fig. 3.—Typical symptoms produced on Yolo Wonder at 28°C. Note systemic necrosis and stem streak.

increased with increase in temperature. By 18 days after inoculation, however, the order of concentrations shifted so that the higher concentrations were at the lower temperatures. The virus concentration in plants at 16 and 20° increased throughout the 18 days of the

Table 2.—TMV concentration 6, 12, and 18 days after inoculation in tip leaves of California Wonder pepper grown at different air temperatures.

Air temperature (°C)	Virus concentration (O.D.)[a]		
	6 days	12 days	18 days
16	0.000	0.084	0.250
20	0.000	0.100	0.120
24	0.160	0.260	0.050
28	0.285	0.060	0.035

[a] Values given are ultraviolet absorbencies (at 265 mμ) of purified preparations on a Beckman DU Spectrophotometer.

Table 3.—TMV concentration 6, 12, and 18 days after inoculation in composite leaf samples of systemically infected plants of Accession 163201-S grown at different air temperatures.

Air temperature (°C)	Virus concentration [a]		
	6 days	12 days	18 days
16	0.100	0.110	0.125
20	0.125	0.242	0.255
24	0.175	0.460	0.210
28	0.210	0.125	0.140

[a] Values given are ultraviolet absorbencies (at 265 mμ) of purified preparations on a Beckman DU Spectrophotometer.

experiment, whereas at 24 and 28° it reached a maximum early and then declined. Both varieties showed the same general concentration pattern among the different temperatures.

The pattern of virus concentrations in inoculated leaves of Yolo Wonder was, in all experiments, identical to that in California Wonder plants. In one experiment the O.D. values of purified preparations made 4 days after inoculation were 0.000, 0.050, 0.095, and 0.260 at 16, 20, 24, and 28°, respectively. In composite leaf samples the virus concentration in all assays increased with increase in temperature (Table 4). This is in marked contrast to susceptible varieties in which patterns were cyclic. In Yolo Wonder, the virus concentration increased steadily in plants at all temperatures throughout the 18 days of the experiment. In tip leaves the virus was isolated only from plants growing at 28°. Even at 18 days after inoculation, no virus was detected in the tip leaves of plants growing at 24° or lower.

As mentioned earlier, only necrotic lesions occurred on inoculated plants of 163201-R and the virus could never be recovered from uninoculated plant parts. Inoculated leaves rapidly abscised following the development of necrotic lesions, and it was considered possible, as suggested by others, that immunity to systemic invasion might merely be that rapid leaf abscission prevented the virus from reaching the stem. To test this hypothesis, stems of plants of 163201-R were inoculated by rubbing with a carborundum-virus preparation, by injection of purified virus into the plant with a hypodermic needle, and by approach and cleft grafts with California Wonder plants. In no case was systemic infection obtained. TMV did not pass from California Wonder plants into plants of 163201-R through a graft union. Histological examination of the graft union showed that the 2 tissues were united by continuous parenchyma.

In a further test, indoleacetic acid (1:1000) in lanolin was applied to the base of inoculated leaves to delay abscission. Though the resulting delay was as much as 6 days, no systemic spread of the virus occurred. In tests with California Wonder and Yolo Wonder in which inoculated leaves were excised at certain intervals after inoculation, data showed that movement from inoculated leaves into stems had occurred within 12 hours at 20° and earlier at higher temperatures. Thus, it would seem that 10 days at 28° would be ample time for the virus to move into the stem. The failure to do so in 163201-R can only be interpreted as being due to complete localization of TMV in the inoculated leaves.

In assays from inoculated leaves of 163201-S and 163201-R at different temperatures at 4 days after inoculation almost no virus was recovered from resistant plants. In one experiment where concentrations in inoculated leaves of 163201-S and 163201-R plants were compared in half-leaf inoculations, the respective average numbers of lesions were: 44 and 1 at 16°, 303 and 2 at 20°, 175 and 6 at 24°, and 142 and 26 at 28°. These low values of virus concentration of samples from resistant plants would indicate that the virus is almost completely inactivated in the lesion tissue, or that lesions are incited by a minimal virus synthesis.

Discussion.—Temperature obviously affects movement of the virus in Yolo Wonder plants since the very tip leaves of plants at 16 and 20° were free of virus even 18 days after inoculation. It is necessary to consider temperature in evaluating breeding material for disease resistance. Crossan and Rahn (2) recently reported Yolo Wonder pepper to be free of symptoms at one month after inoculation in the field. The present studies indicate that this would not be the case at 24°C or above unless the strain of Yolo Wonder they used contained the local-lesion gene *L* described by Holmes (3) and reported herein as occurring in Accession 163201. No Yolo Wonder stock we tested possessed this gene. In their announcement of the variety, Porter and Younkin (6) indicated the absence of the Tabasco local-lesion gene. They implied, however, that inoculated plants later became free of the virus by leaf abscission.

The temperature response of Yolo Wonder pepper to TMV infection is almost the exact counterpart of the reaction of Ambalema tobacco to this virus (1). In both cases, at 16 and 20° the inoculated plants develop very mild symptoms, and the virus concentration in the plants is very low. Disease severity and virus concentration increase with increase in temperature and show a gradient that does not vary with time. There is no evidence of a cyclic concentration pattern, as occurs with this virus in hosts where higher concentrations of virus are reached (1), and virus concentration is markedly lower than in more susceptible varieties. This might add credence to the idea that a

Table 4.—TMV concentration 6, 12, and 18 days after inoculation in composite leaf samples of Yolo Wonder pepper grown at different air temperatures.

Air temperature (°C)	Virus concentration (O.D.) [a]		
	6 days	12 days	18 days
16	0.000	0.000	0.080
20	0.012	0.050	0.115
24	0.060	0.095	0.185
28	0.135	0.245	0.360

[a] Values given are ultraviolet absorbencies (at 265 mμ) of purified preparations on a Beckman DU Spectrophotometer.

cyclic pattern occurs only in host-virus combinations where the virus occurs in high concentration. Pound and Garces (9) recently showed that a cyclic pattern does not occur with the turnip mosaic virus, a virus that also develops in relatively low concentration.

The resistance in Accession 163201 apparently represents immunity to systemic invasion of the virus. Expression of this resistance was independent of the air temperature at which the plants were grown. This is different from the resistance of *Nicotiana glutinosa* to TMV and that of Virginia Savoy spinach to CMV; at high temperatures those plants succumb to a systemic necrosis. The resistance in Accession 163201 apparently is one in which the virus is completely localized to a small area about the point of entry, and in which either the virus synthesized is rapidly inactivated *in vivo* or lesion production results from a minimal amount of virus synthesis.—Department of Plant Pathology, University of Wisconsin, Madison.

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STUDIES ON RESISTANCE IN RADISH TO FUSARIUM OXYSPORUM f. CONGLUTINANS

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SUMMARY

Fusarium oxysporum f. *conglutinans* race 2 penetrates both susceptible and resistant plants of radish through the root tip of primary and secondary roots and through the cortex in the region of differentiation or immediately above. Root hair penetration is infrequent. Invasion through the cortical region was both intercellular and intracellular. The fungus advanced principally in the direction of the stele and was not found to traverse up the cortex to any extent. Susceptible and resistant plants showed little difference in the extent of fungal invasion and amount of mycelium observed in this region. Vascular invasion occurs in both susceptible and resistant plants, but mycelial concentration in the latter was sparse. Deterioration of the stelar and cortical tissues occurred when the cambial region was heavily invaded. Conidial and chlamydospore formation in the xylem vessels was observed only in susceptible plants. Normal thickenings of cortical and endodermal cells were not effective barriers to the fungus, and no suberized thickenings were formed in response to fungal penetration. The fungus was shown to traverse to the upper parts of the susceptible plants, including the leaves and the apical meristem. Resistant plants rarely showed penetration above the lower hypocotyl region. It is believed that resistance is localized principally in the vascular system and that it is inherent to the protoplasm and does not result from any morphological barrier to growth and spread of the fungus. Age of plants had little effect on expression of resistance but concentration of inoculum was a factor in amount of disease development, severity increasing with increased dosage. High concentrations of inoculum markedly reduced the expression of resistance, and stroking the roots of resistant plants before dipping them in inoculum enhanced the effect of the concentrated inoculum. F_1 progenies of susceptible × resistant plants expressed a level of resistance that was intermediate between the parents. The F_2 progenies and backcross progenies showed resistance of about 15%. These ratios suggest that resistance is polygenic in nature.

Introduction.—Fusarium yellows of radish, *Raphanus sativus* L., incited by race 2 of *Fusarium oxysporum* f. *conglutinans* (Wr.) Snyd. & Hans., was described by Kendrick and Snyder (2) in 1942 on Chinese winter radish in California, and later by Snyder and Bardin (5) in commercial plantings of Early Scarlet Globe radish on peat soil in California. A serious disease of radish on muck soils in Wisconsin was shown by Pound and Fowler (4) to be caused by the same organism. This organism is distinguished from the cabbage yellows organism, *F. oxysporum* f. *conglutinans* race 1, on the basis of distinct differences in pathogenicity to cabbage (4). In 1957, the resistant radish variety Red Prince was released to the seed trade (3). This paper reports studies on the nature of the resistance of this variety, and certain factors affecting its expression.

Histological studies.—*Materials and methods.*—Resistant Red Prince radish was developed by selection from the commercial Early Scarlet Globe. Tests of this variety in the greenhouse showed it to be highly resistant in comparison with the susceptible Early Scarlet Globe. Various inoculation techniques were used to study different aspects of infection: 1) planting seeds on artificial media inoculated with the yellows organism; 2) dipping roots of 5-day-old plants in

Table 1.—Infection of young roots and hypocotyls of susceptible Early Scarlet Globe and resistant Red Prince radish by *Fusarium oxysporum* f. *conglutinans* race 2 when grown on agar.

Days exposure to inoculum [a]	Exp. no [b]	Intensity of infection [c] in susceptible (S) and resistant (R) plants													
		Region of differentiation										Vascular area			
		Root Tip		Epidermis		Outer cortex		Inner cortex		Protoxylem		Cortex		Stele	
		S	R	S	R	S	R	S	R	S	R	S	R	S	R
1	1	xxx	xxx	xx	xx	x	xx	0	x	0	x	0	0	0	0
		xxx	xx	xxx	xx	xx	xx	xx	x	x	0	x	0	0	0
	2	xx	x	x	0	x	0	0	0	0	0	0	0	0	0
		x	xx	0	xx	0	x	0	0	0	0	0	0	0	0
2	1	...	xxx	xxx	xxx	xxx	xxx	xxx	xx	xx	x	x	x	x	0
		...	...	xxx	xxx	xxx	xxx	xxx	xx	xx	xx	xx	0	x	0
	2	xxx	xxx	xxx	xxx	xxx	xx	xx	x	x	0	x	0	0	0
		xxx	...	xxx	xx	xxx	x	x	x	0	0	x	0	0	0
3	1	...	...	xxx	xxx	xxx	xxx	xxx	xx	xxx	xx	x	x	x	0
		...	...	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xx	x	x	x	x
	2	...	...	xxx	xxx	xxx	xx	xxx	x	xxx	x	x	0	x	0
		...	...	xxx	xxx	xxx	xxx	xxx	xx	xx	x	x	0	x	0

[a] Young seedlings were placed adjacent to fungus culture in agar plate.
[b] For each of 2 experiments, 2 susceptible and 2 resistant seedlings were examined.
[c] Symbols shown represent infection intensity as follows: 0 = no infection; x = slight infection; xx = moderate infection; xxx = severe infection; ..., not examined, because of root tip deterioration.

an inoculum suspension and transplanting into soil; 3) planting seeds directly into infested soil.

For infection studies on agar media, seeds were first surface-sterilized in mercuric chloride (1:1000), thoroughly washed in 3 changes of sterile water, and germinated on potato-dextrose agar at room temperature. Additional Petri plates containing potato-dextrose agar were inoculated in the center of the plate with inoculum derived from a single-spore culture of the pathogen. The seeds were placed on media the same day as the other plates of media were inoculated with the fungal organism. After 2 days, the seeds with the primary roots extending through the seed coat were transferred to agar plate cultures of the pathogen and were placed around the expanding fungal culture. Care was taken to place the root tip of each seedling at the margin of the developing culture. The primary roots of the seedlings when first placed on the fungus cultures were about 0.5 cm long. As the fungus grew outward, it came in contact with the older regions of the root, containing the root hairs and including the expanding hypocotyl region. The inoculated plates containing the seedlings were incubated at room temperature, and several seedlings were removed each day for 3 days for fixation and embedding.

In soil infection studies at 20–24°C seeds were sown directly in muck soil that had been sterilized and then reinfested. Seedlings were lifted at various intervals for fixation and embedding. In removing the plants, care was taken to lift as much as possible of the entire plant; but even so, some damage to the root system occurred, especially when the plant was severely diseased. The roots were washed by gentle agitation in water.

In other studies seedlings were grown in sterilized muck soil, removed and inoculated when 5 days old, and transplanted into additional sterilized muck soil. After inoculation the plants were kept in soil temperature tanks maintained at 22°C.

Formol acetic alcohol was used as a fixative. A safranin and fast-green combination, as well as Flemings' triple stain, was used for staining the fungus in the tissue. Roots were cut in serial sections 10–18 μ thick. Sectioning of the root continued above the soil line and well above the area of penetration of the fungus.

Relative intensity of infection in resistant Red Prince and susceptible Scarlet Globe Radish.—The results of 2 separate studies of seedlings on agar plates are given in Table 1. These observations were from sections of roots and hypocotyls of both susceptible and resistant plants. There appeared to be the same degree of infection in the root tip and in the differentiating tissues of the roots of both susceptible and resistant plants. In the inner cortical area and the protoxylem vessels of the region of differentiation, however, the resistant plants were less severely infected. Resistant plants also showed less invasion of the stele and cortical region of the differentiated vascular area near the hypocotyl region.

The extent of fungal penetration in susceptible and resistant plants when hand inoculated and planted in sterilized soil is shown in Table 2. Symptoms of yellowing began in susceptible plants about 8 days after inoculation. Root growth of the 2 varieties was about the same until about 12 days after inoculation, when that of the susceptible plants was noticeably reduced. Secondary root development became increasingly reduced in the susceptible variety as time progressed until, in severely infected plants, the production of secondary roots ceased and the plant soon died. The primary roots were brown and decayed at the tip when lifted from the soil. Upon removing plants from the soil it was found practically impossible to remove the entire root tip of the primary root, and the sections invariably revealed that the root tip and region of

Table 2.—Infection in resistant Red Prince and susceptible Early Scarlet Globe radish, hand-inoculated with *Fusarium oxysporum* f. *conglutinans* race 2 and transplanted to sterilized soil.

Days after inoculation	Plant no.	Intensity of infection[a] in: Cortex Susceptible	Cortex Resistant	Stele Susceptible	Stele Resistant
5	1	0	0	0	0
	2	0	0	0	0
	3	0	0	0	0
9	1	x	0	x	0
	2	0	0	x	0
	3	x	0	xx	0
12	1	0	0	x	0
	2	x	x	xxx	x
	3	x	0	x	0
14	1	x	0	x	0
	2	0	0	xx	x
	3	0	0	xxx	x
17	1	x	0	xxx	0
	2	x	0	x	x
	3	0	0	xxx	0
20	1	x	0	xxx	0
	2	x	0	xxx	0
	3	x	0	xxx	x
24	1	x	0	xx	0
	2	x	x	xx	x
	3	0	x	xxx	x

[a] The symbols used denote the degree of infection as evidenced by the presence of the fungal mycelium in the tissues of the root as follows: 0 = no infection; x = slight infection; xx = moderate infection; xxx = severe infection.

differentiation were missing. Secondary roots were found to be more intact, especially in the resistant variety, and the root tips and region of differentiation were easily observed in these.

By the ninth day the fungus had advanced into the stele of the susceptible variety and was also noted in the cortex of the same region (Table 2). The mycelium increased in extent and amount in the susceptible plants and was easily noted in the stele of the primary roots and extending up into the hypocotyl region. Although the cortical cells of the root appeared not to be infected, even though the vascular area in the same region showed the presence of the fungus, they were no longer turgid but appeared to be shrinking and were difficult to section. They often sloughed off prematurely. The resistant plants did not show the presence of the fungus in the root until after the ninth day, and then only rarely was the fungus observed, and always in a low concentration. Secondary root invasion was not often noted, even in susceptible plants. Usually only 2 or 3 roots, including the primary root of susceptible plants and only the primary roots of resistant plants, contained the fungus.

When seeds were planted directly in infested soil, a few susceptible plants showed symptoms of the disease after 5 days, and several had died before the eleventh day (Table 3). The extent of infection of the plants in this test was similar to those inoculated and transplanted into sterilized soil (Table 2).

Pathological anatomy of the radish roots.—1) *Penetration by the fungus.*—Penetration of 2-day-old roots of young seedlings grown on agar was very rapid, generally within the first 24 hours after contact of the roots with the fungus (Table 1). The root tips after penetration became dark, and cells became less turgid and began to deteriorate within 48 hours. The roots grown under these conditions were subject to a very concentrated inoculum, and concentration of the fungus in the root tip was, therefore, generally heavy (Fig. 1-A). Penetration of the root tips was primarily intercellular, but upon entrance into the root cap the fungus rapidly became intracellular as well as continuing to progress intercellularly to the embryonic growing point of the root.

Penetration of the young seedlings was also observed to occur both intercellularly and intracellularly through the epidermal tissue in the region of differentiation and in the upper areas of the root where the protoxylem is very evident. After direct penetration into the epidermal cells the fungus often became intercellular. Penetration through the epidermis occurred to a lesser degree at the level of the well-differentiated vascular area (Table 1). Roots that were naturally infected or hand inoculated had little mycelium in the cortical area of the higher parts of the root (Tables 2, 3) and penetration in these areas was rare. No penetration of the hypocotyl region was observed.

Root hairs were rarely found infected, and in such instances they were usually quite flaccid in appearance and somewhat shriveled.

Secondary roots frequently become infected (Fig. 1-B) as they grow through heavily infected areas of the cortical region, or when they protrude into in-

Table 3.—Infection of resistant Red Prince and susceptible Early Scarlet Globe radish when grown in soil infested with *Fusarium oxysporum* f. *conglutinans* race 2.

Days from inoculation	Plant no.	Intensity of infection[a] in: Cortex Susceptible	Cortex Resistant	Stele Susceptible	Stele Resistant
8	1	x[a]	0	x	0
	2	0	0	0	0
	3	0	x	x	x
11	1	0	0	xxx	0
	2	x	0	xx	0
	3	x	0	xx	0
15	1	x	0	xxx	0
	2	x	0	xx	x
	3	x	0	xxx	0
18	1	x	x	xx	x
	2	x	0	xxx	0
	3	x	0	xxx	0
22	1	0	x	xx	x
	2	x	0	xxx	x
	3	xx	0	xxx	x

[a] The symbols used denote the degree of infection as evidenced by the presence of the fungal mycelium in the tissues of the root, as follows: 0 = no infection; x = slight infection; xx = moderate infection; xxx = severe infection.

fested soil. However, even in sections that showed severe infection of the primary root, the secondary roots were not all infected; in fact, many of the smaller secondary roots were evidently free of mycelium.

The fungus, even when directly penetrating the cells, showed no apparent specialized penetrating structures, i.e., appressoria or infection hyphae. Upon penetration, the hyphae, in comparison to host cells, usually appeared to be somewhat smaller in diameter than before penetration.

No difference could be detected between susceptible and resistant varieties in the initial penetration. Plants of both varieties, when grown on agar cultures, appeared to be easily penetrated through the root tip region and the epidermis of the region of differentiation (Table 1). Penetration through the epidermal areas of the root above the region of differentiation was observed to be less in the resistant variety than in the susceptible variety (Tables 2, 3). There was no observed difference in the mode of penetration of the 2 varieties.

2) *Invasion through the cortical region.*—In the deepest areas of cortical invasion the fungus was generally in an intercellular position, whereas in outer regions it was found to be either intercellular or intracellular. In the studies of the plant roots grown on agar, it was found that even though suberized thickenings may occur on some walls of the cortical cells immediately surrounding the endodermis, these barriers failed to keep the fungus from penetrating deeper.

In cortical regions of the root where the secondary xylem of the vascular system was well formed the infection of the cortex was seldom observed originating from the epidermis. In this region, and in the hypocotyl region, fungal penetration of the cortical cells did occur when the vascular system was severely infected. It was noted in several instances that the inner cortical cells of the hypocotyl region and the older portions of the root became heavily infected with the fungus while the outer cells of the cortex showed no evidence of fungal penetration through the epidermis. In such instances, the mycelium progressed from the vascular region into the surrounding cortical layer (Fig. 2-A). Fig. 2-B is a median longitudinal section in the area proximal to the stele showing the fungus in the cortical cells.

In the upper cortical region the fungus progressed readily between the cells, probably being enhanced by the large intercellular spaces between the parenchyma cells. However, it was not inhibited from being intracellular and it appeared to be equally intracellular and intercellular. As infection of the root became more severe the cortex became more highly invaded, and in some instances conidial formation was observed on mycelium in these severely diseased areas.

3) *Development of the fungus in the vascular region.*—Penetration of the vascular area was mainly via the root tip and the cortical tissue of the region of differentiation, and infrequently in the area of the protoxylem of the upper part of the region of differentiation. The fungus was observed to traverse through the root cap area and into the undifferentiated stele of the root, where xylem vessels were penetrated as they became differentiated. The penetration of the protoxylem vessels was also through the newly formed endodermis and pericycle (Fig. 2-C).

From these areas of original penetration the fungus moved intracellularly in the xylem vessels and was readily observed in upper root areas and the hypocotyl region within 8–9 days (Tables 2, 3). The browning of the vascular area indicated the presence of the fungus in the hypocotyl and fleshy root areas. The fungus was more commonly observed in the xylem vessels (Fig. 1-D) but also in the parenchyma surrounding these cells (Fig. 2-A, B). Some vessels appeared to be entirely plugged with a heavy concentration of mycelium, whereas other cells in the same section showed only a few hyphal strands and some showed no sign of the fungus whatsoever. The fungus appeared to move from cell to cell through the pits in the cell walls and between spiral thickenings (Fig. 2-D). Movement was common from the vessels of the xylem into surrounding parenchyma cells and out into the cortical region. With severe infection the stelar tissue often deteriorated and became difficult to section (Fig. 1-C).

Conidia and chlamydospores were observed in some of the vessels, usually in more advanced stages of the disease (Fig. 1-E).

The only differences observed between resistant and susceptible varieties in the development of the fungus in the vascular system was in the extent and concentration of the fungus in the stele. The concentration of mycelium in the upper region of the roots and hypocotyl was greater in the susceptible variety than in the resistant variety (Tables 2, 3).

4) *Reaction of the host to the presence of the fungus.*—No morphological difference could be detected between roots of resistant and susceptible plants. Upon infection the root cells in some instances appeared to show a protoplasmic reaction to the presence of the fungus. Cells of the epidermis and the immediately underlying cortex showed plasmolysis and sometimes a granular substance not seen in healthy tissue. The plasmolysis and granulation were not common to all infected cells of these layers and appeared in both resistant and susceptible plants grown and infected on agar plates. There was no apparent indication of a shriveling or granulation of mycelium within these cells.

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Fig. 1.—Pathological anatomy of radish roots infected with fusarium: **A)** Root tip of 3-day-old resistant plant showing heavy concentration of intercellular and intracellular mycelium. **B)** Young secondary root showing mycelial penetration. **C)** Cross section of root of 45-day-old plant showing breakdown in cambial region. **D)** Longitudinal section of susceptible root showing network of mycelium in a pitted vessel. **E)** Longitudinal section of a 30-day-old root showing conidial and chlamydospore production in the vessel walls.

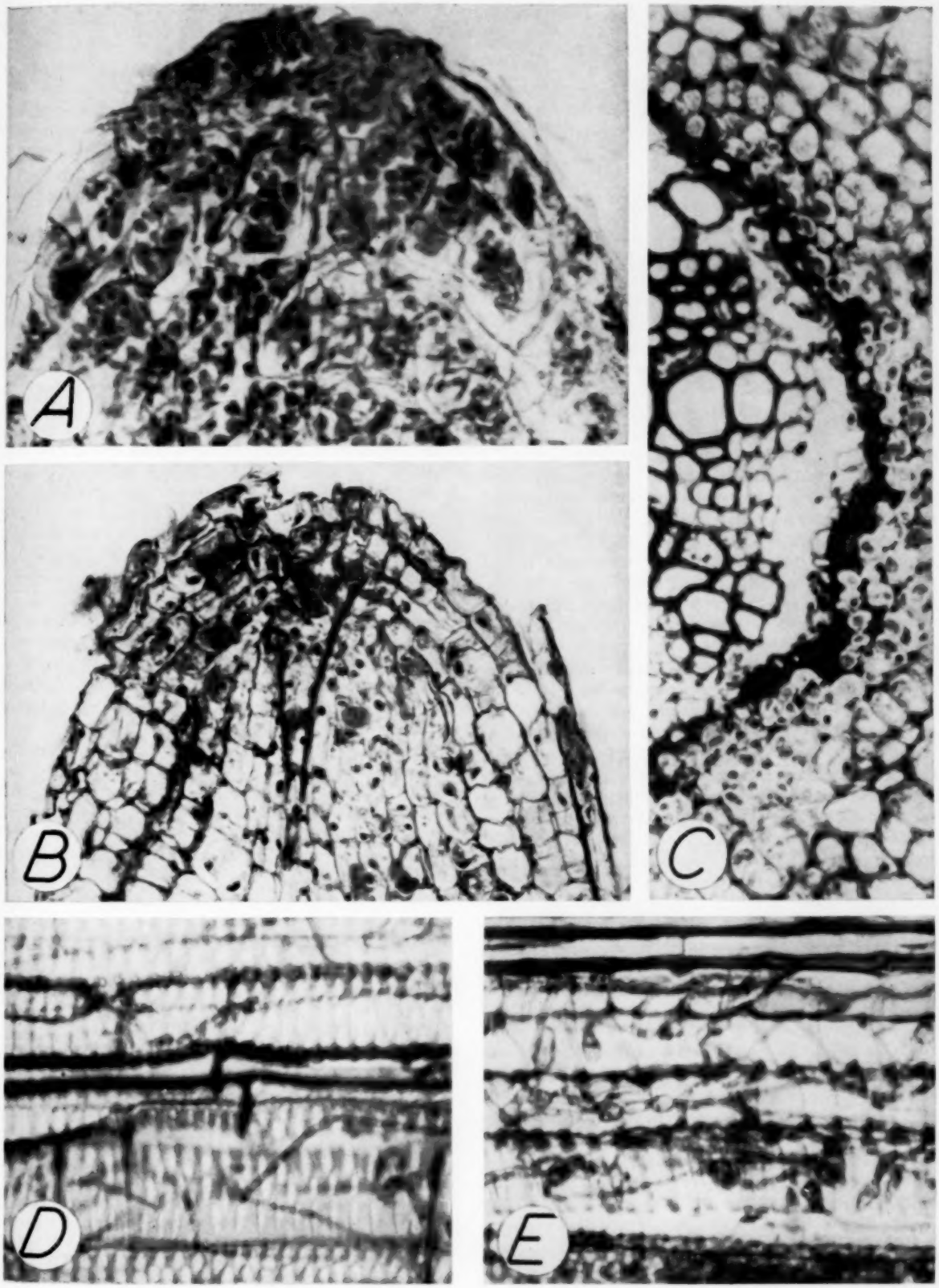
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Table 4.—The occurrence of *Fusarium oxysporum* f. *conglutinans* race 2 in various parts of resistant Red Prince and susceptible Early Scarlet Globe radish.

Plant part	Susceptible		Resistant	
	No. plants tested	% plants infected	No. plants tested	% plants infected
Root tip	14	93	11	73
Root	27	82	19	42
Root-hypocotyl	23	91	16	13
Hypocotyl	27	89	19	0
Lower stem	27	78	19	5
Upper stem	11	64	6	0
Apical meristem	27	52	17	0
Leaf petiole[a]	12	83	5	0
Leaf blade[a]	10	80	1	0
Cotyledon	2	50	2	0

[a] From leaves growing near the base of the stem.

Although normal suberization occurs in the endodermis and some surrounding cortical cells, this did not appear to be effective in stopping the advance of the fungus into the stelar region. The fungus became intracellular and penetrated these cells that showed suberization, particularly on the radial walls. There was no indication in either radish variety of a suberization in response to the stimulation or presence of the fungus that would inhibit further fungal penetration. Tyloses were observed to occur in the vessels of resistant as well as susceptible plants, and whether the root was infected or not.

The fungus was observed to penetrate the provascular area and also the endodermis in areas of the protoxylem and, with concentrated inoculum, into the stele in areas where metaxylem formation had taken place. No invasion was observed in areas above these, and the fungus readily moved up the vascular area, apparently effectively contained within the region of the stele. When the infection became more severe the fungus finally penetrated through the pericycle and endodermal layers into the cortex. Although mycelium was not always found in the cortex when the vascular area was heavily infected, a browning of the cortex was often noted with no mycelium being present. The cells were less turgid than normal and consequently were difficult to section. Higher sections of the same roots in areas where the fungus was not present in the vascular system exhibited an apparently healthy cortical layer.

Extent of fungal penetration in the aboveground parts of the plant.—Plants of both resistant and susceptible varieties grown in infested soil or sand were carefully washed and cut into serial sections that were surface-sterilized and plated on agar. Some of the plants had developed flower stalks. The fungus was isolated from stem parts as well as the apical meristem area of many of the susceptible plants (Table 4). In the few samples taken of leaf tissue it was also possible to isolate the organism from leaf blades and petioles. In resistant plants the fungus was often isolated from the root and the lower part of the hypocotyl, and browning of the vascular system was noted in these areas. Vascular browning was conspicuous in upper stems and leaves of susceptible plants. The fungus was usually isolated slightly above vascular browning, which was visible to the unaided eye. Several free-hand cross sections were made of the lower stem areas, and the fungus was noted to be present only in the vascular area of the tissues observed.

Relation of plant age to resistance.—Snyder and Bardin (5) reported that radish plants observed in field plantings varied in development in relation to the disease. Many roots reached marketable size, and of this group some showed internal symptoms of the disease while other plants developed to intermediate degrees between the marketable size and little or no growth of the plant. This would indicate that there may be a difference in the resistance of plants in respect to age as well as heritable differences in resistance.

Three experiments were performed to test the relative resistance of plants of varying ages in both the resistant and susceptible varieties. In the first study 5 plantings of seeds were made in soil at 10-day intervals. At 3 days after the last seeding, the plants were lifted with care, and the washed roots were dipped into a mycelial suspension of the organism and planted into pans containing sand watered with Hoagland's nutrient solution. The pans were then placed in soil temperature tanks where the sand temperature was maintained at 22°C. A second experiment was run under the same conditions except that seedlings were started in sand rather than in soil and seedlings were at 9- rather than 10-day intervals. The oldest plants in both tests were generally developing seed stalks, some over 4 in. long, while the youngest were in the cotyledon stage, 3 days old. In the third experiment, plantings in crocks containing quartz sand were made every 6 days for a total of 6 plantings. When the youngest plants were 2 days old, a 40-ml solution of a concentrated mycelial suspension was poured on the surface of the sand and was then watered in with nutrient solution. The survival rates of the susceptible variety were about the same in all age groups.

The age of the plant at inoculation had little effect on the ultimate amount of infection, and this effect was not altered by the method of inoculation. In the first experiment the susceptible variety at 22 days after inoculation showed 75% and 90% for plants that were

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Fig. 2.—Pathological anatomy of radish roots affected with fusarium: **A)** Longitudinal section of upper root area showing mycelium advancing from vascular region into cortical tissue. **B)** Longitudinal section through the cortex, proximal to the stele, showing intracellular mycelium that originated in the stele. **C)** Cross section of a 3-day-old susceptible root growing on agar and showing mycelium within the cells of the endodermis and pericycle. **D)** Cross section of a 16-day-old susceptible root showing mycelium penetrating from vessel to vessel.

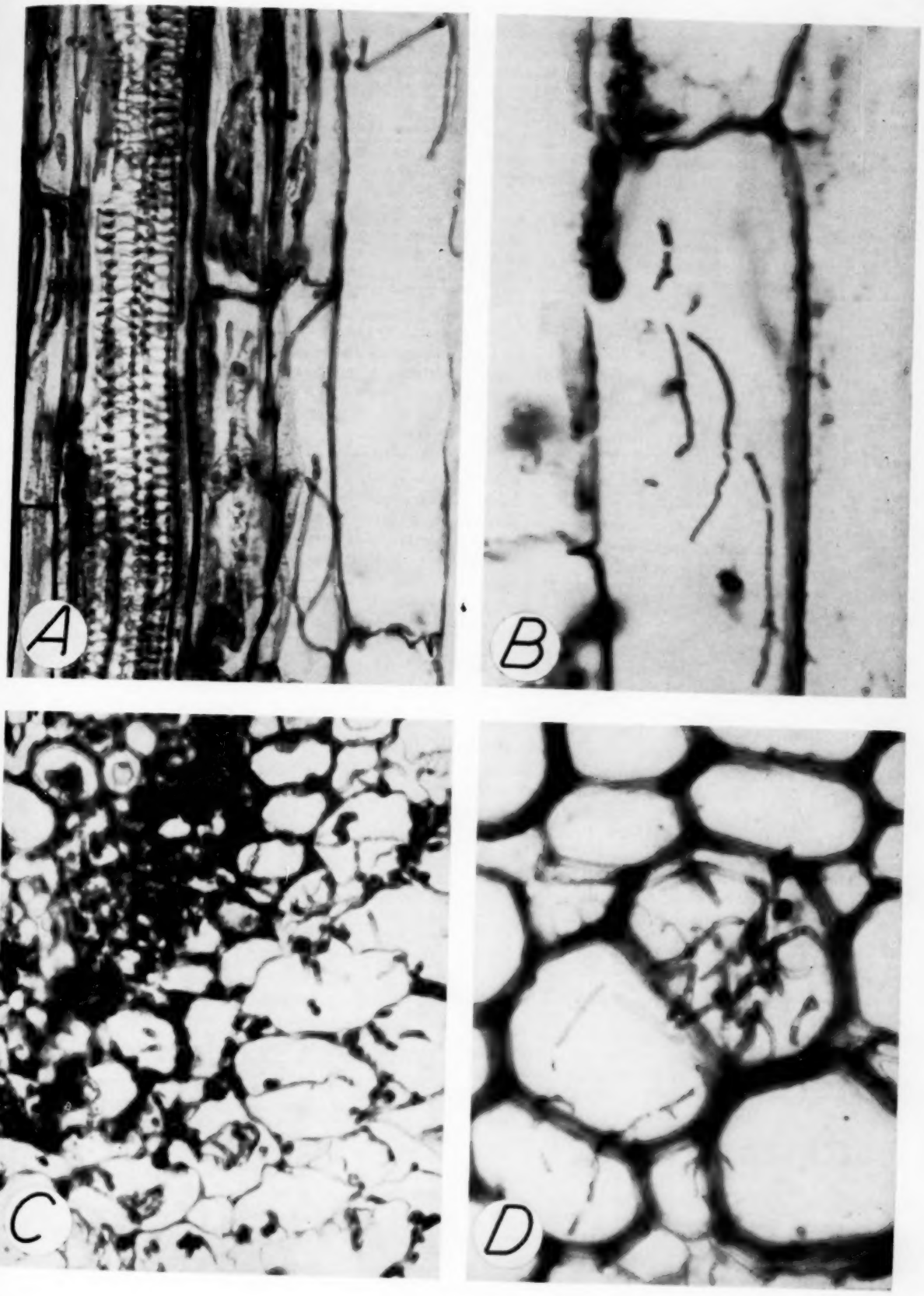
A
B
C
D

Table 5.—The effect of inoculum dosage and inoculation technique on the amount of yellows in resistant and susceptible radish plants.

Method of inoculation	Inoculum dilution	Percentage of diseased plants of 28 inoculated after interval shown							
		Resistant plants				Susceptible plants			
		6 days	8 days	11 days	14 days	6 days	8 days	11 days	14 days
Roots-unstroked	Undiluted	17.9	42.8	46.4	50.0	14.3	67.8	100.0	100.0
	1:1	10.7	21.4	25.0	25.0	3.6	53.6	96.4	100.0
	1:3	0	0	0	0	0.0	53.6	92.8	100.0
	1:7	0	0	0	0	3.6	57.1	85.6	100.0
Roots stroked	undiluted	35.8	78.5	89.3	89.3	14.3	85.6	100.0	100.0
	1:1	17.9	25.0	25.0	25.0	10.7	78.5	89.3	100.0
	1:3	3.6	7.2	7.2	7.2	7.2	60.7	96.4	100.0
	1:7	0.0	0.0	14.3	14.3	3.6	42.8	92.8	96.4

respectively 3 days old and 39 days old at inoculation. In the second experiment the corresponding values for the same age groups were 90% and 88%. The third experiment yielded similar results. Resistance of Red Prince was expressed quite uniformly at all age groups, with the percentage of infected plants ranging from 0 to 19.

Relation of inoculum dosage and method of inoculation to amount of infection.—In preliminary inoculations it was noted that heavy concentrations of inoculum caused a quick and severe disease reaction in both susceptible and resistant plants, and that stroking the roots by hand before dipping them into the fungal suspension increased the severity of the disease reaction in both types of plants.

Three experiments were performed to clarify the effect of inoculum dosage. Plants were inoculated, with and without hand-stroking of the roots, with various dilutions of an inoculum prepared either from agar plates or from liquid media. The data in Table 5 give the results of an experiment using inoculum produced in liquid media where the undiluted inoculum consisted of the fungus mats of a dozen 800-ml bottle cultures (200 ml in each) homogenized in 150 ml water. In susceptible plants there was little effect from stroking the roots prior to inoculation. In resistant plants, however, stroking the roots markedly increased the amount of disease, especially with the undiluted inoculum. Inoculum dosage was a factor with both types of plants. In resistant plants the amount of disease after 14 days was much greater with the undiluted inoculum than with the diluted inoculum. In susceptible plants the undiluted inoculum did not affect the ultimate amount of disease with either method of inoculation, but where stroked plants were inoculated the rate of disease development was markedly increased.

Inheritance of resistance.—Resistant Red Prince radish was developed by selection within the Early Scarlet Globe variety from plants that had survived in a severely affected field (3). To study the inheritance of this resistance, crosses were made with susceptible plants of Early Scarlet Globe and White Icicle. All resistant plants used in crossing had been previously inoculated in the seedling stage. All pollinations were made under controlled conditions, and all flowering branches were covered with bags to reduce the chance of contamination by foreign pollen. In crosses between resistant and susceptible plants and in backcrosses to the susceptible parent all blossoms were emasculated and hand-pollinated. Since radish is highly self-infertile, little seed set resulted from self pollination. The F_1 progenies of the resistant and susceptible crosses were assayed in pan tests, with bulk seed of the resistant and susceptible varieties included as controls.

The F_1 progenies varied considerably in amount of disease as well as in rate of disease development. Crosses with Early Scarlet Globe and White Icicle showed very similar reactions. The progenies were all distinctly intermediate in disease reaction between the 2 parents (Table 6). Some lines showed a rela-

Table 6.—The occurrence of Fusarium yellows in F_1 progenies from crosses between resistant Red Prince and susceptible White Icicle varieties.

Progeny	Total plants	Percentage of diseased plants at intervals after inoculation				
		7 days	10 days	14 days	17 days	20 days
1 (F_1)	83	7	12	40	49	52
2 (F_1)	77	0	7	32	47	47
3 (F_1)	57	2	18	37	68	68
4 (F_1)	28	21	36	39	61	64
5 (F_1)	80	4	30	41	51	60
6 (F_1)	80	11	34	38	49	56
7 (F_1)	75	0	3	21	40	41
8 (F_1)	28	32	51	64	79	86
9 (F_1)	125	11	22	39	58	66
Red Prince	16	0	0	0	0	0
White Icicle	17	18	47	100	100	100

tively rapid dying of diseased plants, whereas others showed a relatively slow progression of disease. Some plants died before full development of their first true leaves, whereas others showed disease symptoms in their true leaves and were, in some instances, still alive at the termination of the test.

Several F_1 plant selections that apparently remained healthy in the screening tests in the greenhouse were backcrossed to the susceptible parental lines. The progenies from these backcrosses were then tested in the greenhouse for indications of the amount of resistance in the plants. In the F_2 populations derived from the Early Scarlet Globe backcrosses there appeared to be from 7–30% resistant plants, whereas the White Icicle backcross populations were 5–20% resistant. Some lines appeared to go down more rapidly than others, and several plants in certain lines appeared only slightly diseased. One line was 30% resistant and relatively slow in developing disease symptoms. Many of these plants were still alive at 20 days, even though showing disease symptoms.

Discussion.—The response of resistant Red Prince radish to infection by the yellows organism has been shown to differ from the susceptible Scarlet Globe variety. This difference in the pathological anatomy is in degree rather than kind of infection, and was more explicit in histological studies of plants taken from soil than in studies of plants grown on agar, where disease was more severe.

Penetration of the 2 varieties occurred in a similar manner, and apparently of the same degree. Root tip penetration occurred both intracellularly and intercellularly in both varieties. Upon entrance into the root tip, the fungus apparently proceeded unhindered into the lower region of differentiation, where it no doubt was able to penetrate cells of the vascular system as they became differentiated. The fungus in this region appeared to be as intracellular as it was intercellular. It was also able to gain access to the radish root by penetrating the epidermis of the region of differentiation. Epidermal penetration was both intracellular and intercellular, the fungus using no specialized penetrating mechanism to gain entrance. After entering the cortical region from the epidermis, the more rapid progress was through the intercellular pathways. This type of progress was indicated since the mycelium observed in areas of the deepest penetration was generally intercellular. The mycelium, however, can rapidly become intracellular and in this way penetrate past the suberized layers often noted in the inner cortical region of many roots and through the endodermal layer into the differentiating stele. Penetration through root hairs was rare. Thus it is believed that root hairs are of only minor importance as an avenue of fungal entrance.

Studies indicate that the radish fungus in both varieties fails to spread to any extent in the cortical areas of the root above the region of differentiation after initial penetration through the epidermis. It was also observed that penetration of the epidermis in the hypocotyl region did not occur and was also rare in the upper root area. The normal cutinization of the epidermal cells of the hypocotyl region, plus one or more layers of sub-epidermal cells (1) could act as a barrier to fungal invasion in this region.

Penetration studies of plants grown on agar indicated that the fungus could move rapidly through the cortical region into the differentiating stele. In some instances entrance into the stele was accomplished within 2 days of root contact with the fungus. Upon entrance into the stele the fungus became principally located in vessels of the xylem, where it progressed upward into regions of well-differentiated xylem tissue. It was not limited to the vessels, however, but was observed in the xylem parenchyma, in the cambial region, and in the phloem region. Several instances of cambial disorganization and deterioration were observed in response to the presence of the fungus. This was indicated by heavy staining and tearing of cells. When the vascular area was severely affected with the fungus, mycelium was observed to have progressed from the vascular area into the cortical region. It is believed that this is the principal cause of the cortical deterioration or browning of the upper root and hypocotyl, rather than a cortical progression of mycelium from the root tip area or direct penetration in this region from the epidermis. Even when the fungus was not evidenced in the cortex, there was still cortical deterioration in some roots. This deterioration was a loss of turgidity and browning of the cortical region, and was observed to occur in several instances when the vascular system was heavily infected. Upper sections of the same root, when the fungus was not observed in the vascular region, exhibited a cortex free of mycelium. This deterioration of the cortex may be a physiological response to the presence of the fungus in the vascular system.

In some instances in plants grown on agar, there was a cellular response to the presence of the fungus. The epidermal and immediately underlying cortical cells showed plasmolysis and sometimes a granular substance not seen in healthy tissue. This response, however, was not common to all plants infected, and cannot be considered as a resistance reaction. The fungus in these cases was not inhibited from further penetration, and there was no apparent effect, such as granulation, on the penetrating mycelium. There was no evidence of suberization of any cells in response to the fungus.

The results suggest that resistance is based on the inability of the fungus to develop within the host to an extent that can cause severe injury. In some instances mycelium was observed in the xylem of the resistant variety, but in these cases it was very sparse. Although the penetration into the stele can occur, the upper areas of the hypocotyl and stem are somehow restricted. It appears that the principal resistance to Fusarium yellows may be located within the vascular tissue. These conclusions are further substantiated by the studies of the extent of fungal penetration in the aboveground parts of the plant. Culturing specific portions of the plant showed that the fungus was able

to traverse nearly the whole extent of the susceptible radish plant. It was isolated from leaves, upper parts of the stem, and the apical meristem of several plants. The fungus rarely traversed higher than the upper root or lower part of the hypocotyl of resistant plants. Browning of the vascular system appears to be a result of the presence of the fungus, since the fungus was isolated in the areas of principal browning.

Different concentrations of inoculum were shown to have a marked effect on the development and severity of the disease. More dilute suspensions provided a good differential between the 2 varieties, whereas the more concentrated suspensions tended to produce disease symptoms in the resistant as well as the susceptible variety. The symptoms produced by these heavy concentrations differed somewhat from the normal disease symptoms. Plants that showed severe atypical symptoms usually failed to develop any further. Stroking roots of seedlings prior to dipping them in inoculum resulted in little change in disease reaction in the more dilute concentrations, but distinct increases in severity in higher concentrations. Winstead and Walker (6), studying toxic metabolites in relation to Fusarium resistance, showed that replacement filtrates from the radish wilt organism would cause symptoms similar to those of infected plants. They suggested that the organism produced metabolites that have a specific effect on susceptible varieties and in some way may aid establishment of the pathogen. This toxic reaction may be the partial cause of the atypical symptoms produced early in plants by concentrated mycelial suspensions. Wounding the roots by stroking may make possible or accentuate the toxin effect in resistant plants.

Studies of crosses between selected plants of resistant Red Prince and plants of susceptible Early Scarlet Globe and White Icicle varieties indicate that the resistance of Red Prince is governed by modifying factors and that these factors are cumulative in effect. The F_1 progenies of the resistant $\times$ susceptible crosses showed considerable variation in the degree of resistance. In the crosses with Early Scarlet Globe the resistance of the F_1 progenies was generally above 50% whereas those with White Icicle showed a slightly lower level of resistance. If resistance were controlled by several factors, the level of resistance would be substantially lower in the F_1 than in the resistant parent. This was the case. Studies with backcrosses of F_1 plants to the susceptible parents showed a considerable drop in resistance from that expressed in the F_1 progenies. Early Scarlet Globe backcross progenies showed 7–30% resistance, and White Icicle backcross progenies showed 5–20% resistance. Selfed progenies from F_1 plants of the Early Scarlet Globe susceptible and resistant crosses also showed a low percentage of resistant plants. F_2 and backcross progenies from these crosses showing a resistance level of around 15% would indicate a polygenic inheritance.

The variation in resistance in F_1 and F_2 progenies can be explained on the basis of segregation of factors. Resistance in the F_1 and F_2 generations was greater in the Early Scarlet Globe crosses than in the White Icicle crosses. This can be explained by the presence of a smaller number of factors for resistance in Early Scarlet Globe. In testing for resistance, it is noted that Early Scarlet Globe varied in the development of the disease and showed slightly more resistance than White Icicle.

Although Red Prince appeared to be highly resistant, a few plants became diseased in the progenies of selfed plants. It is believed that the resistant variety is not completely homozygous in factors responsible for resistance.—Department of Plant Pathology, University of Wisconsin, Madison.

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TETRIN, AN ANTIFUNGAL ANTIBIOTIC

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SUMMARY

Tetrin is a new antifungal agent produced by a *Streptomyces* sp., Ill. No. 155-2. The antibiotic is a polyene with 4 unsaturated conjugated double bonds, fluoresces under ultraviolet light, and has absorption maxima at 290, 305, 319 mμ in aqueous solutions. It can be differentiated from other tetraenes by various characteristics, including solubility in solvents, movement on paper chromatograms, stability to pH, biological activity, and other criteria. The crystalline antibiotic sinters but does not melt even at 360°C and has an empirical formula of $C_{31}H_{59}O_{12}N$. The crude antibiotic is soluble in water, methanol, ethanol, dimethyl formamide, pyridine, and methyl cellosolve; it is insoluble in aliphatic, and aromatic hydrocarbons as well as acetone and ether. Tetrin is stable at neutral and alkali pH, but loses activity under acid conditions. One hour at 100°C caused no loss in activity.

Though many antifungal antibiotics have been synthesized by microorganisms, relatively few of them have found wide use for plant protection against microbial diseases. This situation is in marked contrast to the intensive application of antibacterial antibiotics in human and animal therapy. Such a paucity of antibiotics for combating fungal diseases is probably correlated with the relatively few materials studied for this purpose. Intensive investigations are needed to unearth new antibiotics as well as to determine the usefulness of those already described. Because many surface protectants are available for plant disease control, needs are for substances that can be translocated in plants and are systemically active. Tetrin, found in such a program, appears not easily translocated, but it is relatively nontoxic to plants, and might be effective in plant disease control.

Materials and methods.—*Streptomyces* sp. isolations were made from soil (collected at Felton, California) suspended in Petri plates. Initial dilutions were made in distilled water and final dilutions in Emerson's agar (distilled water, 1000 ml; yeast extract, 1.0 g; beef extract, 4.0 g; Bacto-peptone, 4.0 g; sodium chloride, 2.5 g; cerelose, 10.0 g; agar, 20 g). Preliminary antibiotic activity determinations were made on the same medium by the conventional method of streaking the streptomycete on an agar plate, allowing it to incubate 10 days at 26°C, and then counter-streaking the test organisms perpendicular to the original streak. Synthesis of the antibiotic in various liquid media was studied on a reciprocal shaker with a 4-in. stroke and 110 strokes per minute. A common inoculum for use in all of the different media in a single experiment was made by adding sterile water to a slant of the streptomycete, rubbing the spores off the mycelium, and inoculating with the suspension a flask of Emerson's medium. Incubation on the shaker for 2 days produced enough mycelium to inoculate the various media. Shake flask studies on the synthesis of tetrin were later carried on in a similar manner.

Assay plates were prepared by putting a base layer of 20 ml of tryptocase soybean agar into Petri plates; after it had hardened, 6 ml of the same agar containing 4% of a *Penicillium oxalicum* spore suspension were poured on the base layer. Spore suspensions were prepared by growing the fungus on test tube agar slants (V8 juice, 200 ml; calcium carbonate, 3 g; agar, 20 g; distilled water, 800 ml) for seven days; 5 ml of a 0.01% aqueous solution of Vatsol was added to each tube, and the surface of the culture was gently rubbed with a nichrome transfer loop. This suspension was diluted with sterile distilled water until 50% light transmission was obtained when measured at 530 mμ in a Lumetron Colorimeter. Similar plates were prepared with *Microsporum canis*, *Fusarium oxysporum* Schlecht., *F. lycopersici*, *Trichophyton interdigitale*, *Glomerella cingulata*, and *Verticillium* sp. Schleicher and Schull 13-mm discs were placed on the agar, and 0.08 ml of solution was pipetted on the discs. The plates were incubated for 24 hr, and the zone of inhibition was measured. Antibiotic potency of culture fluid or of the various extracts and solid preparations of the antibiotic was based on a dilution unit. The unit is defined as the greatest dilution in ml of 1 ml of a preparation in Czapek's agar (sucrose, 30.0 g; sodium nitrate, 2.0 g; potassium dibasicphosphate, 1.0 g; potassium chloride, 0.5 g; magnesium sulfate, 0.5 g; ferrous sulfate, 0.01 g; distilled water, 1000 ml) that would inhibit the growth of *P. oxalicum* for 72 hr when a suspension of spores of 50% transmission was streaked on the surface of a slant containing the antibiotic. Tetrin Preparation V was used as a standard, and its innate potency was determined by incorporating various concentrations in Czapek's agar at increments of 1 μg/ml. Growth was inhibited at a minimum of 20 μg/ml. The standard preparation thus contained 50 dil. units per mg. Antibiotic potencies of other preparations were determined by using concentrations from 62 to 1000 μg/ml of Tetrin Preparation V as a basis for concentration-inhibition zone standard curve. The diameter of inhibition zone was plotted as a function of concentration. Inhibition zone diameters that were produced by the unknown were interpolated on the standard curve and their respective potencies expressed in dilution units.

Ultraviolet absorption studies were made on either a Beckman DU or Cary Recording Spectrophotomer. Paper chromatograms were made by applying solutions of the antibiotic in 0.01 ml portions to sheets of Whatman No. 1 filter paper cut to 14 x 21 in. The

Table 1.—Inhibition of microorganisms by Streptomycete Ill. No. 155-2.

Microorganisms	Distance (mm) of linear inhibition from streak
Aspergillus niger van Tiegh.	17
Fusarium lycopersici (Brushi) Wollenw.	16
Glomerella cingulata (Stonem.) Spauld. & Schrenk	25
Microsporum canis Bodin	25
Penicillium oxalicum Currie & Thom	17
Trichophyton interdigitale Priestley	18
Verticillium sp.	17
Candida albicans (Robin) Berkh.	7
Mycoderma cereviseae Desm.	15
Saccharomyces cereviseae Meyen ex Hansen	15
Saccharomyces globosus Osterw.	13
Saccharomyces pastorianus Hansen	12
Torula sp.	17
Zygosaccharomyces priorani Klocker	14
Alcaligenes faecalis Castell. & Chalmers	0
Bacillus cereus Frankland & Frankland	0
Klebsiella capsulate (Sternberg) Bergey et al	0
Micrococcus aureus Zopf	0
Mycobacterium tuberculosis 607 Zopf	0
Pseudomonas aeruginosa (Schroet.) Migula	0
Sarcina lutea Schoet.	0

spots were allowed to dry between each application, and total quantity varied with the antibiotic activity of the sample. The ascending solvent flow system was used, and further treatment was carried out as described by Ammann et al (1). The solvent systems were: 1) water-saturated butanol; 2) n-butanol–acetic-acid–water (2:1:2); 3) n-butanol–pyridine–water (1:0.6:1; 4) 3% ammonium chloride; 5) 50% aqueous acetone; 6) benzene–acetic-acid–water (2:2:1); 7) n-butanol–methanol–water (2:2:1); 8) benzene–methanol–water (1:1:2); 9) 70% propanol; 10) n-butanol–ethanol–water (5:1:4). *P.-oxalicum*-seeded trays were used to detect the position of the antibiotic on the paper, and UV lamps located it by the fluorescence due to the conjugated unsaturated bonds. Attempts at extraction and purification utilized standard chemical methods such as differential-solubility fractional-precipitation with various solvents; alumina, carbon, and magnesium carbonate absorbants; ionic and cationic resins; and elution from paper chromatograms.

All experiments involving plants were done in the greenhouse. Plants (Bonny Best tomatoes, Detroit Dark Red beets, Crimson Giant radish, Early Green Sprouting broccoli, Hawkeye soybean, Black Seeded Simpson lettuce, Allbig sweet pepper, Southport Yellow Globe onion, Florida Market eggplant, Wisconsin Early Sweet garden pea, Hales Best No. 36 muskmelon, and Burpee's Stringless Green Pod beans) were grown in sterile soil in 4-in. pots. For toxicity and protection studies, 4-week-old plants (3 to a pot, with 3 replicate pots) were sprayed with an aqueous solution of tetrin by a deVilbiss hand sprayer until the leaves were completely wetted. Control plants were sprayed with water. Toxicity readings were made for 3 weeks following spray application. Studies of the effect of the antibiotic on seed germination were made by placing the seeds into Petri plates on filter paper that had been saturated with the antibiotic and covering them with a similarly treated paper. Antibiotic solutions were added as needed to keep free solution in the Petri plate at all times. In other experiments the seeds were soaked for 3 hours in solutions of tetrin and then allowed to drain on filter paper before being placed in Petri plates containing filter papers saturated with distilled water. Five replicated plates were used for germination studies, and per cent of germination was recorded each day for 6 days. Systemic toxicity was determined on 2-month-old Bonny Best tomato plants that had been removed from pots, the soil washed from the roots, and then placed in solutions of antibiotic of 1000 μg/ml or in water. Final observations were made after 1 week.

The translocation of tetrin was studied with 3 procedures: A) One leaf of cucumber plant was immersed in a vial containing 500 μg of antibiotic per ml. After 5 days the antibiotic concentration in plant parts above and below the leaf was assayed by squeezing out the juice with a garlic press. B) Bean plants with the trifoliate leaves emerged were washed free of soil and the roots immersed in solutions of various tetrin concentrations for 2 days. The crowns of the plants were then cut off well above the solution, frozen at −40°C, and thawed, and the juices were extracted with a press for assay. C) Pads of cheesecloth soaked in antibiotic solutions were used to completely wet lower leaves of plants with two such treatments 24 hr apart. Water was used as the control treatments, and triplicates of 3 plant sets were used for all treatments. Assays were made using plant juices that were obtained as described above.

Plant protection studies on asparagus rust were made by spraying Martha Washington plants with tetrin solutions of 0, 500, and 1000 μg/ml. The young branches and cladophylls were then dusted with urediospores of *Puccinia asparagi* in a 1:20 urediospore-talc mixture. The plants were kept 24 hr in a humidity chamber and placed on the greenhouse bench. Infection counts were made at the end of 15 days.

Results.—*Production and isolation of the antibiotic.*—Tetrin, an antifungal antibiotic having four unsaturated conjugate double bonds, was isolated from the liquid growth medium of a *Streptomyces* sp., Ill. 155-2 (isolated from soil collected at Felton, California). This organism was chosen for streak-plate criteria because of its ability to inhibit the growth of various yeasts and mycelial fungi (Table 1). Neither the streptomycete nor the antibiotic derived from its culture inhibited any of the bacteria tested. Streak-plate activity occurred on a modified Emerson's medium but this medium was not used by the streptomycete for synthesis of the antibiotic in liquid shake culture. Of the many shake-culture media tried, tetrin was synthesized only in Abbot-Complex medium (distilled water, 100 ml; brown sugar, 10.0 g; lactose, 5.0 g; dextrin 151, 5.0 g; yeast 2019, 2.0 g; curbay

Table 2.—Antibiotic production by Streptomycete Ill. 155-2 in liquid media.

Fungi	Diameter (mm) of inhibition zones [a]
Microsporum canis Bodin	28
Fusarium lycopersici (Brushi) Wollenw.	23
Trichophyton interdigitale Priestley	28
Penicillium oxalicum Currie and Thom	27
Glomerella cingulata (Stonem.) Spauld. and Schrenk	30
Verticillium sp.	27

[a] Assay on Tryptocase-Soy agar plates seeded with the fungus.

B.G., 2.0 g; B-Y fermentation solubles, 5.0 g; ammonium nitrate, 2.0 g; corn steep liquor, 2.0 ml; calcium carbonate, 4.0 g; sodium chloride, 5.0 g; glycerol, 5.0 ml; adjust pH to 7.0) and Kelner-Morton medium (distilled water 1000 ml; bactopeptone, 5 g; glucose, 10 g; molasses (Brer Rabbit Green Label), 20 ml; $FeSO_4 \cdot 7H_2O$, 0.01 g; adjust pH to 7.0). All antifungal inhibition observed on streak plates also occurred when the culture medium was tested on assay plates, each containing a different test fungus (Table 2). *P. oxalicum* assay plates were used routinely for all subsequent studies.

The antibiotic was stable to alkali for 24 hr at pH 11 in the culture medium in which it was produced, but it lost 76% of the original activity when kept at pH 2.0 for that period. Tetrin was fairly stable to heat, and even after 60 min. at 100°C retained 78% of its original activity. Autoclaving at 121°C for 15 min. resulted in a 50% loss. Filtration through a Seitz filter resulted in a 25% loss, whereas passage through a sintered glass UF filter reduced activity only 12%.

Early recognition of the polyenic nature of the antibiotic resulted from a study of the ultraviolet absorption spectrum of the brew. That it contained 4 conjugated double bonds was obvious from the position of the three UV absorption maxima obtained (Fig. 1). The name "tetrin" was chosen because of the presence of 4 double bonds.

The best extractant for removing the tetrin from Kelner and Morton shake flask culture was n-butanol. One-half volume of solvent to one of medium removed all but a trace of the antibiotic as detected on assay plate tests, and a second treatment at ¼ volume completed the extraction. Attempts at extraction failed with chloroform, ether, benzene, petroleum ether, and ethyl acetate. The active principle in the medium was absorbed by activated carbon, Darco G-60, and Nuchar C-115, but elution of the antibiotic was unsuccessful with methanol, 70% methanol, acetone, 80% acetone, 0.1*N* sodium hydroxide, 0.1*N* hydrochloric acid, and 0.1*N* methanolic hydrochloric acid. Other agents that did not remove tetrin were hexane, ether, n-propanol, benzene, low-boiling petroleum ether, 0.1*N* methanolic potassium hydroxide, and methyl isobutylketone. Dimethylformamide eluted 22% of the absorbed antibiotic from the Nuchar but not from the Darco carbon; methylcellosolve and pyridine, on the other hand, removed it from both absorbants. Ethanol was also somewhat effective. Successive treatments of the carbons with ethanol followed by methylcellosolve removed 65–90% of the absorbed antibiotics in "batch" treatments. The ion exchange resin IR-120 also removes tetrin from solution; elution with 10% sodium chloride releases 20% of the antibiotic that was removed from the shake culture fluid.

The best antibiotic preparations were obtained by inoculating an aqueous spore suspension of *Streptomyces* 115-2 into 100 ml Kelner and Morton media in 500-ml Erlenmeyer flasks and incubating 2 days in shake culture to produce inoculum. Three ml of this inoculum was used as "seed" to inoculate other flasks. After 6 days on the shaker, the cultures were harvested and the mycelium separated from the culture fluid by centrifugation in a basket centrifuge. Twenty L of the medium was extracted with 22 L of n-butanol. The solvent was evaporated in a rotary evaporator under reduced pressure at 44°C. Ethanol was added to dissolve the antibiotic from the residue, and 4 volumes of ethyl ether were added to the alcoholic solution to precipitate the antibiotic. The precipitate was dissolved in cold water and freeze-dried. The yield, 20.73 g of a light-tan flocculant powder, contained 41.5 dilution units per mg.

Properties of the compound.—Studies by Prof. K. L. Rinehart, Jr., and Mr. V. F. German, Department

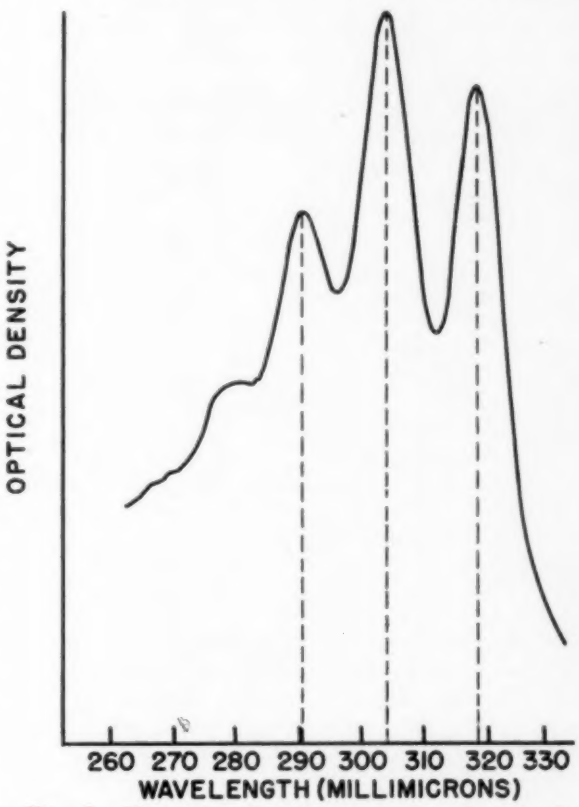

Fig. 1.—Ultraviolet absorption spectrum of tetrin in ethanol (14.4 ug/ml).

of Chemistry and Chemical Engineering, University of Illinois, resulted in further purification of the active material. Craig counter-current separations and subsequent precipitations of the active fractions gave a crystalline compound that sinters but does not melt even at 360°C. Elemental analysis showed the presence of only carbon, hydrogen, oxygen, and nitrogen. From the percentage of these elements in the antibiotic, a tentative empirical formula was calculated as $C_{31}H_{59}O_{12}N$. Further investigations on the structure and chemical properties of tetrin are under way.

Tetrin is one of the few water-soluble polyenes. It also dissolves in methanol, pyridine, sodium hydroxide, hydrochloric acid, methyl cellosolve, and diformamide, and is slightly soluble in chloroform. Macroscopic quantities are insoluble in hexane, benzene, acetone, low-boiling petroleum ether, ethyl ether, and methylisobutylketone. In aqueous solution, tetrin was entirely inactivated in 16 hr at pH 2.0, but showed no decrease in activity during the same period at pH 6.5 or 12. Aqueous solutions lose some activity even when stored at low temperatures; concentrations of 250 and 500 μg/ml lost 20% activity in three months at 1°C. The antibiotic reacts with concentrated sulfuric acid to produce a blue color that is characteristic of many polyenes.

The ultraviolet absorption spectrum of tetrin is characterized by the presence of three peaks with maxima at 290, 305, and 319 mμ and a shoulder at 280 mμ (Fig. 1). The antibiotic fluoresces when exposed to ultraviolet light, and paper chromatographic studies show that this property is associated with the antibiotic spot R_F.

The R_F values for the movement of substances on paper chromatograms can never be stated in precise terms unless experimental conditions are closely controlled. They vary with temperature, degree of saturation of environment of the paper, and many other factors. This close control was not possible in these experiments. Nevertheless, R_F values can be applied to the differentiation of chemicals when they are run at the same time and under identical conditions. When used in this manner, paper chromatography was of value in differentiating tetrin from some of the other polyenes. The ranges of R_F for tetrin in various solvent systems for 3–6 different experiments are given below. Water-saturated n-butanol (0.15–0.22), n-butanol–acetic-acid–water (0.68–0.77), n-butanol–pyridine–water (0.64–0.75), 3% ammonium chloride (0.5–0.69), 50% acetone (0.82–0.90), benzene–acetic-acid–water (0.00), n-butanol–methanol–water (0.64), benzene-ethanol-water (0.00), 70% propanol (0.65–0.69), n-butanol–ethanol–water (0.38–0.55).

Comparison to determine its individuality.—The literature was surveyed to determine whether tetrin had been known previously. No compounds with identical characteristics could be found. Whenever possible, authentic samples of the other tetraenes were obtained and compared to tetrin on the basis of paper chromatographic behavior, biological activity, and other properties.

That amphotericin A (6, 13) was not identical with tetrin was indicated by the following facts. Amphotericin A is only one-third as active against *Cryptococcus neoformans* as against *Microsporium canis*. Tetrin, on the other hand, was one-seventh as active on *M. canis* as on *C. neoformans*. Amphotericin A was inactivated by alkali, whereas tetrin was stable. Furthermore on paper chromatograms mixtures of these antibiotics separate completely in solvent systems 1, 2, and 4. Antimycoin (11) was ruled out because, in agar plates, its activity against *Aspergillus niger* was less than one-twentieth of that of tetrin. Its spot of biological activity on chromatograms did not fluoresce. Chromin (15) was separated because it was insoluble in water and the lower alcohols that dissolved tetrin and because of its lability in alkali. Mycomycin (2) was not similar, for it inhibited bacteria as well as fungi whereas tetrin is restricted to antifungal activity. The solubility properties as well as its marked instability, with explosive decomposition, rules out any identity between these two antibiotics. Nystatin (4, 7) is also distinct from tetrin by its insolubility in water and the slight solubility of the new antibiotic in these agents. Furthermore, mixtures of these antibiotics were separated on paper chromatograms with solvent systems 1, 2, and 3. Antibiotic PA 166 (5, 8) was readily differentiated from tetrin by its insolubility in water. Identity of tetrin with rimocidin (3, 16) was ruled out by the separation of the 2 materials on paper chromatograms using solvent system 4. Tetrin proved to be different from pimaricin (14) on the basis of water insolubility as well as its biological activity. The concentration required to inhibit *Trichophyton interdigitalis* was 25–100 times as great as that required to inhibit most other fungi. This marked resistance to the fungus did not occur with tetrin.

Protocidin (12) was shown to be different from tetrin on the basis of R_F values on paper chromatograms in solvent 1; furthermore, protocidin produced a brown coloration when treated with concentrated sulfuric acid, whereas tetrin formed a blue coloration. Crystalline protocidin is reported to melt at 120–121°C (12), whereas tetrin begins to sinter only at 150°C

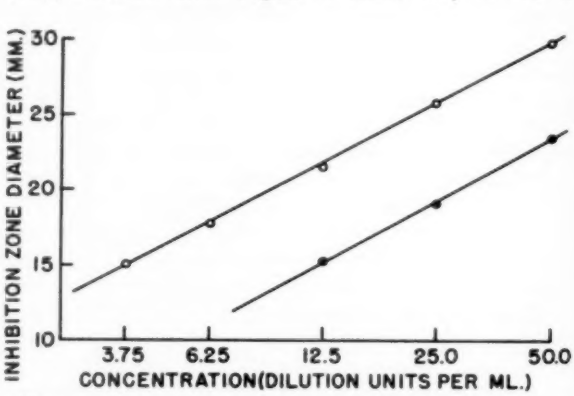

Fig. 2.—Tetrin assay curve on *P. oxalicum* plates: open circles, one layer flat bottomed; filled circles, regular, two layer.

Table 3.—Concentration of tetrin required to inhibit growth of fungi in Czapek's agar dilution plates.

Organism	Concentration (dilution units per ml)
Candida albicans (Robin) Berkh.	10.0
Saccharomyces cereviseae Meyen ex Hansen	2.5
Cryptococcus neoformans (Sanfel.) Vuill.	0.6
Microsporum canis Bodin	1.1
Fusarium lycopersici (Brushi) Wollenw.	2.5
Trichophyton interdigitale Priestley	0.6
Penicillium oxalicum Currie & Thom	1.1
Aspergillus niger van Tiegh.	1.1
Ceratostomella ulmi Buism.	0.6
Penicillium chrysogenum Thom	0.6
Aspergillus fumigatus Fresen.	1.1

and melted with decomposition at 160°C. Sistomycosin (9) could not be confused with tetrin because the former is labile whereas the latter is stable. When treated with concentrated sulfuric acid, sistomycosin produces a red to brown color, whereas tetrin produces the typical blue color of many polyenes. Furthermore, sistomycosin had a 0.5 R_F more than tetrin in 3 solvent systems. Tetrin could not be confused with Tennecitin because when the two antibiotics were put on papergrams and developed in a butanol–ethanol–water (5:1:4) solvent system, tetrin always had a slightly greater R_F, i.e., respectively .53 and .41. Tennecitin is a yellow amorphous powder, whereas tetrin is white.

Assay procedures.—Dilution assays are more sensitive than diffusion zone inhibition methods. Various dilutions of the antibiotic in Czapek's agar were tubed as slants, allowed to stand overnight, and inoculated with spores of *P. oxalicum*. Preparation 5, used as a standard, inhibited the growth of the fungus for three days at 20 μg/ml but not at 19 μg/ml. One mg of preparations therefore contains 20 dilution units.

The potencies of all other preparations of antibiotic were determined by assaying them on the basis of the standard preparation by the agar plate seeded inhibition zone technique. The following method resulted in the most sensitive assay. *P. oxalicum* was grown on V-8 agar (distilled water, 800 ml; V-8 Juice (Campbell Soup Co.), 200 ml; calcium carbonate, 3.0 g; bacto agar, 20 g) slants to obtain inoculum for the assay plates. Spores were removed after 7–10 days by adding a 1:10,000 solution of vatsol in distilled water to the slant and rubbing the surface colony lightly with a glass rod. The density of the spore suspension was adjusted to 50% light transmission; trypticase-soy agar was the medium for the seeded assay plates. Four ml of spore suspension were added to 100 ml of this medium and kept at 45°C after sterilization. Five ml of the seeded agar was placed on the bottom of each "flat bottomed" Petri plate. To obtain a standard reference curve, suitable aqueous dilutions of the antibiotic were prepared to give concentrations from 125 to 1000 μg/ml. Schleicher and Schuell 13-mm assay discs were placed on the seeded agar, and 0.08 ml of the solution was added to the disc. Zones of inhibition were read after 18 hrs. Various dilutions of the culture fluids or different "preps" were placed on similar discs, and the antibiotic activity was determined in dilution units based on the standard prep. Plotting the concentration on a logarithmetic scale and ring size on an arithmetic scale resulted in a straight-line graph (Fig. 2).

Tetrin is a broad-spectrum antifungal agent. It prevented growth of all the fungi tested, but of none of the bacteria. Eleven fungi with which quantitative studies were made using semi-crystalline preparation (50 dilution units per mg), were inhibited at concentrations between 0.6 and 10 dilution units per ml (Table 3). The fungi used were plant pathogens, animal pathogens, and saprophytes.

Description of culture.—Isolate Ill. 155-2 is a typical member of the genus *Streptomyces*, with a substrate mycelium and aerial hyphae. It has spiral sporophores and would thus belong in section spira according to Pridham's classification (10). The gray color of its sporulating hyphae on many agar media further limits it to one of the organisms in the gray series. No specific epithet has yet been assigned to the isolate. Detailed characterization on various media follows: Plain gelatin—colorless vegetative growth, no aerial growth, complete liquefaction. Nutrient gelatin—colorless vegetative growth, trace white aerial growth, complete liquefaction. Tryptone broth—trace colorless vegetative ring at surface, flocculent at base, very slight trace white aerial growth. Litmus milk—colorless vegetative ring, trace pink aerial growth, no change. Nutrient nitrate broth—trace colorless ring at surface, flocculent at base, trace white aerial growth, no reduction. Synthetic nitrate broth—compact vegetative growth at base, no reduction. Calcium malate agar—colorless vegetative growth, gray white aerial growth. Glucose asparagine agar—colorless vegetative growth. Peptone iron agar—colorless vegetative growth, no H_2S darkening. 0.1% tyrosine agar—pink-tan vegetative growth, gray aerial growth, pink tan pigment. Maltose tryptone agar—cream vegetative growth, pale gray aerial growth. Casein starch agar— colorless vegetative growth, pale gray aerial growth, starch-hydrolyzed. Nutrient starch agar—colorless vegetative growth, pale gray aerial growth, starch-hydrolyzed.

Table 4.—Carbon assimilation pattern of Streptomyces Ill. 155-2.

Positive	Positive (slight growth)	Negative (slight growth)	Negative
D-xylose	L-arabinose	dulcitol	phenol
rhamnose	D-fructose	salicin	cresol
D-galactose	D-mannose	Na formate	Na salicylate
D-glucose	dextrin	Na oxalate	
maltose	D-sorbitol	Na tartrate	
sucrose	Na acetate		
lactose	Na citrate		
cellobiose	Na succinate		
raffinose			
inulin			
soluble starch			
glycerol			
D-mannitol			
DL-inositol			

Skim milk agar—yellow vegetative growth, partial hydrolysis. Bennett's agar—good growth, gray aerial growth. Czapek's sucrose agar—good growth at 18° and 24°, fair at 28°, none at 37°, colorless vegetative growth, gray aerial growth.

Plant studies.—Tetrin is not toxic to tomato, beet, pea, radish, broccoli, and soybean plants when sprayed with concentrations of 250–1000 μg/ml. On soybean, even 2000 μg/ml produced no abnormal effects. These concentrations did not inhibit the germination of pepper, broccoli, onion, or eggplant. Germination of radish and lettuce was reduced about 75% in one test, but not at all in subsequent experiments.

Translocation of the antibiotic could not be detected in the juice from cucumber or tomato plants treated with tetrin by immersing one of the leaves in a solution of 500 μg/ml and then analyzing the other plant parts. When bean roots were placed in solutions of tetrin, no antibiotic could be detected in the upper plant parts by the disc assay. Nevertheless, some toxic effect was observed in the curling and drying of the tips of the secondary leaves at concentrations of 1000 and 2000 μg/ml. This symptom could be indicative of movement of the antibiotic in the plant. Movement of tetrin into the leaf from its surface was observed only at high antibiotic concentrations. Leaves of beans to which the antibiotic had been applied for 2 consecutive days had no antibiotic in the expressed juice at treatment levels of 250–1000 μg/ml. After treatment with 2000 μg/ml, however, 11 dilution units per ml of juice was found in the treated leaves, but none in any other of the plant parts.

Tetrin did not control asparagus rust. Instead, it appeared to stimulate the disease. Treatments of 0, 500, and 1000 μg/ml respectively resulted in 11, 25, 16 sori. Another experiment confirmed these results. *Cercospora sojina* was controlled on soybean, though results were erratic. The 250 and 2000 μg/ml treatments reduced infection 16% whereas the 500 and 1000 μg/ml levels respectively reduced infection 40 and 44%.—Department of Plant Pathology, University of Illinois, Urbana.

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METHODS FOR DETERMINING THE REACTION OF SWEET POTATO LINES TO SOIL ROT

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SUMMARY

A reliable method was developed for testing pathogenicity of *Streptomyces* isolates on sweet potato. This consisted essentially of dipping fresh, small roots in a suspension of the organism, embedding them in moistened vermiculite, and incubating 5–7 days at 32°C. A positive correlation was shown between the number of layers of non-nucleated periderm and the resistance of sweet potato to the soil rot organism, *Streptomyces ipomoea*. The more resistant sweet potato lines had fewer layers of non-nucleated periderm. It was also shown that sweet potato lines with higher rates of root development were generally more resistant to soil rot.

Introduction.—The sweet potato (*Ipomoea batatas* (L.) Lam.) breeding program under the leadership of Dr. H. B. Cordner of the Department of Horticulture at the Oklahoma Agricultural Experiment Station has produced many thousand sweet potato seedlings to be evaluated for their relative value as potential new sweet potato varieties. One of the objectives

of the program has been to produce sweet potato varieties that, in addition to other desirable characters, are resistant to as many diseases as possible. Among the diseases considered in this program has been soil rot incited by *Streptomyces ipomoea* (Person & Martin) Waks. & Henrici. Soil rot has not been of major importance as a sweet potato disease in Oklahoma, but it is common and is of particular significance in drier years and in sandy soils.

That resistance to soil rot is available in sweet potato has been demonstrated by Watson (6), Martin (4), and Struble (unpublished work at the Oklahoma Agricultural Experiment Station). One of the more difficult problems of breeding for resistance to this disease has been ready identification of resistance or susceptibility. Although a variety of techniques have been tried for evaluating resistance, it is still necessary to resort to field tests in infested soil to determine the reaction of sweet potato lines. Field tests, though perhaps ultimately necessary, are, for a screening program, too time-consuming and too subject to vagaries of weather to be consistently reliable.

The present investigation was conducted to evaluate several possible techniques for determining the reaction of sweet potato lines to soil rot. Only techniques that show promise are reported. A summary report of these techniques has been published (1).

Test for pathogenicity.—Among greenhouse or laboratory tests tried for distinguishing resistance and susceptibility to soil rot in sweet potatoes were several dependent upon the relative amount of rootlet rot and top growth that results from inoculations under controlled conditions. In other tests attempts were made to inoculate and evaluate severity of soil rot on mature and on small roots. Another test involved an attempt to correlate the relative chlorogenic acid content of roots with resistance. It was possible to distinguish differences between sweet potato lines with respect to their relative susceptibility to soil rot with most tests tried, but the differences were not considered sufficiently distinct and consistent to be of real value in a screening program.

Preliminary to tests involving inoculation to determine varietal reaction, it became necessary to establish a reliable technique for determining pathogenicity of *Streptomyces* isolates and maintaining them in a stable condition. A variety of techniques for determining pathogenicity were tried, including one reported by Person and Martin (5). For the most part, these techniques were dependent upon a discoloration of roots for positive results. In general, reactions, even with a known pathogenic culture, were so variable and unreliable as to make results questionable.

In the course of testing techniques that might be suitable for differentiating resistance or susceptibility, a method adapted from the work of Lawrence (3) with *Streptomyces scabies* (Thaxt.) Waks. & Henrici was found to be very good for testing pathogenicity of *Streptomyces* isolates from sweet potato. This method involved the use of small (3-6 mm in diameter), freshly dug, soil-rot-susceptible sweet potato roots cut into pieces about 6 cm long. These were washed in sterile distilled water and dipped into a chopped mycelium-spore suspension of the *Streptomyces* isolate in question. The root pieces were then embedded in sterilized vermiculite in 145×20-mm Petri dishes and incubated 5-7 days at 32°C. Before autoclaving, the volume of vermiculite necessary to fill one of these dishes was moistened with 100 ml of distilled water.

This technique gave consistent, positive results with pathogenic cultures. Pin-point lesions were evident 3 days after inoculation, and well-formed lesions 5 days after inoculation (Fig. 1). *S. ipomoea* was easily reisolated from unbroken lesions on these roots.

This technique made it possible to show that repeated transfers of pathogenic cultures on a synthetic medium resulted in a gradual loss of pathogenicity. This loss of pathogenicity, previously suspected, had not been demonstrated previously because techniques were inadequate. The effect on pathogenicity of repeated transfers on Czapek's agar is illustrated in Fig. 1. The best way to eliminate this loss in pathogenicity was through periodic transfer of the desired culture from a stock culture maintained in a soil-sand mixture. Cultures have been reisolated from 2-year-old soil-sand stock cultures without evident loss of pathogenicity.

The technique presented here for testing for pathogenicity proved effective but was not useful for evaluating differences in susceptibility between sweet potato lines.

Periderm test for resistance.—In an attempt to identify resistance to common scab (incited by *S. scabies*) in white potato (*Solanum tuberosum* L.), Cooper et al (2) discovered that resistance was associated with the relative number of non-nucleated cell layers in the periderm of tubers. Resistant lines

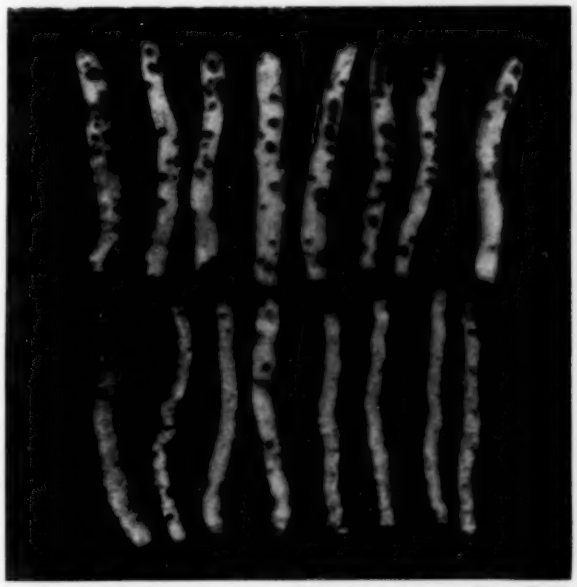

Fig. 1.—Roots of a susceptible sweet potato line, P-97, showing relative amounts of infection when inoculated with a pathogenic isolate, 58-40, of *Streptomyces ipomoea*, upper row; roots in lower row inoculated with a similar isolate, 58-M-1, after 4 transfers on Czapek's agar.

Table 1.—Comparison of ratings of field reaction to soil rot with mean layers of non-nucleated periderm on roots of 6 sweet potato lines with statistical significance as shown by Duncan's multiple range test.

Line	Field rating [a]	Mean layers [b] of non-nucleated periderm for each indicated root: 1	2	3	Mean
Nemagold	R	3.00	2.75	3.00	2.92
Acadian	R	3.75	3.75	3.75	3.75
Allgold	I	5.25	3.25	4.00	4.17
4-126	S	4.50	4.50	5.00	4.67
P-97	S	5.75	5.50	5.00	5.42
Unit #1 Porto Rico	S	7.25	7.00	6.50	6.92

Statistical Significance [c]

Nemagold	Acadian	Allgold	4-126	P-97	Unit #1 Porto Rico
2.92	3.75	4.17	4.67	5.42	6.92

[a] R = resistant, I = intermediate, S = susceptible.
[b] Each figure is a mean based on four observations.
[c] Any 2 means not underscored by the same line are significantly different at the 5% level.

had a few or no non-nucleated layers whereas susceptible lines had several such layers.

In the present work, an attempt was made to correlate resistance to soil rot in sweet potato lines with the relative number of layers of non-nucleated cells in the periderm of roots. Free-hand sections of root periderm were stained with aceto-carmine (2) and the layers of non-nucleated periderm were counted.

Data on the non-nucleated periderm from mature roots of 6 sweet potato varieties or lines used in this work are presented and compared with ratings of reactions of these lines to soil rot in field tests in Table 1. Three roots of each line were used, and, for each root, counts were made at each of 4 locations. From these data it will be noted that there was statistical significance between lines with respect to the relative number of non-nucleated periderm layers. The more resistant a line was, the fewer the non-nucleated periderm layers. There was a high degree of correlation between ratings based on non-nucleated periderm and ratings based on data with these lines planted in infested field soil. Differences in periderm were also present in immature roots 5–10 mm in diameter. The difference between 2 sweet potato lines relative to cell layers of non-nucleated periderm in roots 5 mm in diameter is illustrated in Fig. 2.

The validity of this periderm test for identifying resistance or susceptibility to soil rot in sweet potato was further tested by making counts of layers of non-nucleated periderm from 23 sweet potato lines in addition to the 6 noted in Table 1. According to the number of non-nucleated cell layers found, each line was rated as resistant, intermediate, or susceptible in its expected reaction to soil rot. Again there was a high degree of correlation between non-nucleated periderm ratings and ratings from field tests with these lines. Of the 29 sweet potato lines tested, the range in mean layers of non-nucleated periderm layers was from 1.25, in Heartogold (resistant), to 9.5, in L 3-77 (susceptible).

The principal difficulty involved in evaluating reaction of sweet potato lines was where, either from the periderm test or field data, a line had been designated as intermediate in reaction. In a few cases were a line had been designated as intermediate by one of the tests, the other test showed it to be either resistant or susceptible. It appears that the intermediate category is not clear-cut, which is borne out by several years of field testing that demonstrated that many sweet potato lines are not clearly either highly resistant or very susceptible.

On the basis of evidence presented that there is a high degree of correlation between resistance to soil rot and a fewer number of non-nucleated periderm layers, it seems that the periderm test should be useful in evaluating sweet potato lines for their reaction to soil rot.

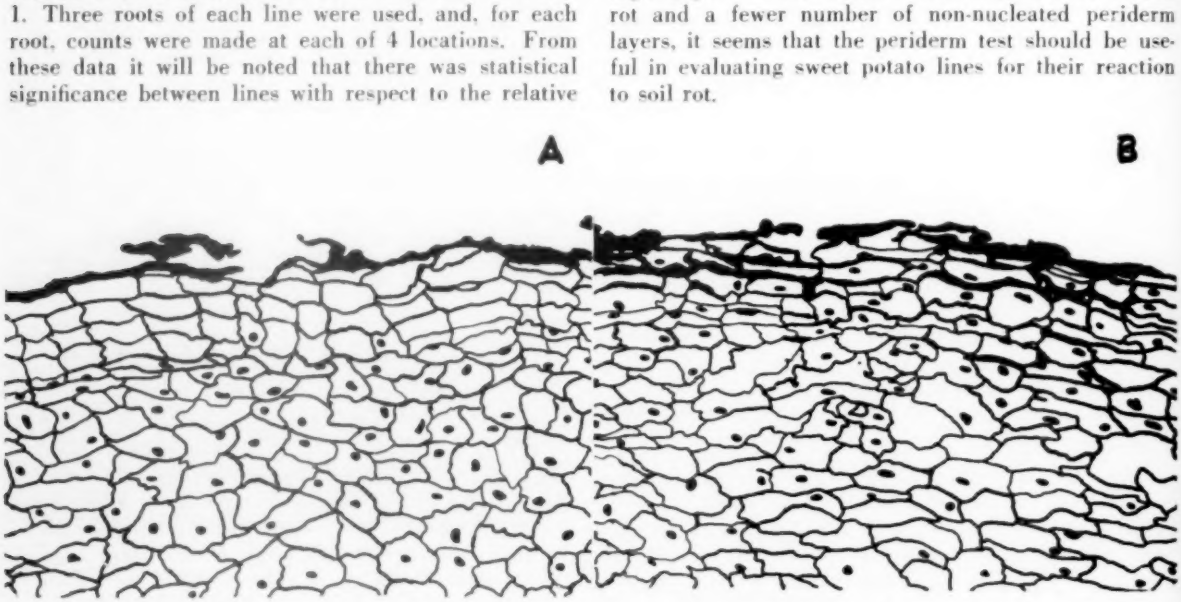

Fig. 2.—Comparison of 2 sweet potato lines with reference to non-nucleated periderm layers on roots 5 mm in diameter. **A)** P-97, a soil-rot-susceptible line. **B)** Acadian, a soil-rot-resistant line.

Table 2.—Root development in each of 12 sweet potato lines as related to soil rot reaction.

Line	Av. number of roots	Av. length per root (cm)	Relative root development	Field rating
Acadian	18.00	5.00	90.00	R*
Nemagold	18.88	4.11	77.60	R
P-81	16.00	4.40	70.40	I
4-126	22.00	3.10	68.20	S
4-107	8.20	6.00	49.20	R
Okla. 61	11.67	3.83	44.70	I
Redgold	8.10	3.75	30.38	I
Allgold	4.50	6.10	27.45	I
P-97	11.00	2.30	25.30	S
Okla. 51	7.80	2.50	19.50	I
P-108	5.90	2.35	13.87	S
Unit #1 Porto Rico	8.40	1.50	12.60	S

* R = resistant; I = intermediate; S = susceptible.

Root development and resistance.—From experience with tests of sweet potatoes for their reaction to soil rot it had been observed that some of the more resistant lines apparently produced more roots, and longer individual roots, than did susceptible lines. Martin (4) has noted this phenomenon in the relatively resistant variety, Heartogold. In an attempt to determine if this ability was consistently associated with resistance in the present work, 10 cuttings each of 12 different sweet potato lines were rooted for 1 week in water. Then the roots of each cutting were counted and measured. To get a single value for root development for each line, the average number of roots per cutting was multiplied by average root length. The product was designated as a value representing relative root development for each line. Results from this test on relative root development are compared with field ratings of these lines in Table 2. Differences in root growth between 2 sweet potato lines are illustrated in Fig. 3. Generally, the more resistant lines have an appreciably higher root development value than the susceptible lines. Higher root development values do not in all cases coincide with resistant field ratings. This can be explained by the fact that the field ratings, for the most part, are based on readings from mature roots, and mature root resistance is not necessarily correlated with resistance to the rootlet rot phase of soil rot. It will be noted from the data in Table 2 that a resistant line may have either a greater number of roots per plant or the average root length may be greater, or both may occur.

There is evidence, then, that the more roots a given sweet potato line produces, and the more rapidly they grow, the more likely the line may be resistant to soil rot.

Discussion.—The periderm layers were found to be more readily distinguishable in fresh roots. While readings could be made from stored roots, nuclei in the cells of the periderm of these tend to become more difficult to see distinctly. Specifically how many non-nucleated layers are necessary to make a line resistant can not be stated with present knowledge. On the basis of evidence at hand, though, it seems that a line with fewer than 4 non-nucleated layers in mature roots should have some resistance. Generally young or immature roots had a layer or 2 less of non-nucleated periderm than did mature roots. Comparisons of lines should be based on roots at approximately the same stage of development.

Since a causal relationship between non-nucleated periderm and resistance or susceptibility has not been established, it might be that this is simply a chance correlation. It is suggested that one way in which non-nucleated periderm may function is to allow *S. ipomoea* to become established more readily where there are several layers or to make it more difficult for *S. ipomoea* to become established where there are fewer layers. The relative amount of senescent or dead tissue may be a determining factor in the establishment of this organism preliminary to its activities as a pathogen.

At present there is no evidence to demonstrate how an abundant root system allows a plant to be more resistant. It is suggested that with such a root system a plant is better able to survive after loss of some rootlets. Martin (4) suggested that an abundant root system allows a plant to escape the early effects of rootlet rot. There is at least observational evidence to indicate that apparent resistance to the rootlet rot phase of this disease does not necessarily mean that older or mature roots may not be severely attacked.

A proper evaluation of resistance would seem to require consideration of both the non-nucleated periderm and root development.

As pointed out by Martin (4) and noted repeatedly in the Oklahoma investigations, the resistance generally available in present sweet potato lines or varieties is not of a very high type. This may explain the repeated failure to identify resistance satisfactorily by any kind of test involving inoculation of rootlets or more mature roots.—Department of Botany and Plant

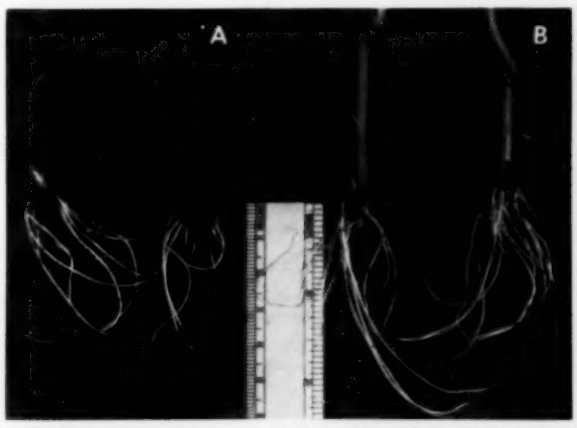

Fig. 3.—Comparison of root systems of 2 sweet potato varieties. Note that Allgold (A) has fewer and shorter roots than Nemagold (B).

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FACTORS AFFECTING ZOOSPORE PRODUCTION BY APHANOMYCES EUTEICHES

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SUMMARY

Cultures of *Aphanomyces euteiches* isolate 50, grown for 5 days in maltose-peptone broth, produced more zoospores when the medium was replaced with tap water for 1–2 hours followed by distilled water, than with other water combinations. Tap water alone produced fewest zoospores. When 30 or 50 ml of distilled replacement water were used, more zoospores were obtained with 5-day-old cultures than with cultures 3, 7, 9, or 11 days old. When 20 ml of distilled replacement water was used, concentrations were highest with cultures 3 days old. Numbers of zoospores of isolates 50, 72, and 103 were largest when temperature of the distilled replacement water was 24°C. The optimum culture temperature range for zoospore production of isolate 50 was 20–28°. Vegetative growth was maximum at 28°. Under optimum conditions 5-day-old cultures of isolates 50, 72, and 103 usually produced $2–3\times10^5$ zoospores per ml of distilled replacement water. Aeration of distilled replacement water containing mycelium increased zoospore production 2–4 times by isolate 50, and 6 or more times by isolate 102, normally a poor zoospore producer. Presence or absence of light had no effect on zoospore production by isolate 50.

The search for parent varieties of pea resistant to common root rot (*Aphanomyces euteiches* Drechs.), and the testing of breeding lines segregating for resistance and susceptibility to this disease, require inoculation in the greenhouse with large numbers of zoospores (3). Since the number of zoospores produced by cultures of the organism varied from test to test, possible sources of this variability were studied. A brief report has been published (2).

Several media, including pea decoction (1, 6), maize kernel decoction (4, 5), Pablum (4), and maltose-peptone broth (3), have been used in culturing *A. euteiches* for zoospore production. Generally zoospore production has been induced by washing mycelium with several changes of "water" (1, 6) or tap water (4, 5). Distilled water or solutions of NaCl, $MgSO_4$, or sucrose were inferior to tap water as sporulating media (5). Temperature optima for zoospore production have been reported to be 15–20°C (4) and over the range of 4–32° (5).

Materials and methods.—Cultures of 4 isolates of *A. euteiches*, each derived from a single zoospore, were used. Isolate 50 was from Michigan; isolate 72 was supplied by W. T. Schroeder, New York Agricultural Experiment Station, Geneva; and isolates 102 and 103 were supplied by L. O. Lawyer, California Packing Co., San Leandro. Inoculum was prepared by growing the fungus 4–5 days in Petri dishes on potato-dextrose agar (per liter: extract from 200 g potatoes; 20 g agar; 20 g dextrose). Uniform disks of mycelium cut with a cork borer were used to inoculate flasks of liquid media.

The method used to induce zoospore production was similar to that described by Smith and Walker (6). The fungus was usually grown 5 days at 23–25° in 50 ml of maltose-peptone broth (per liter: 3 g maltose; 1 g peptone) in acid-washed 250-ml Erlenmeyer flasks. The mycelial mats were left in the same flasks, the medium was poured off, and about 60 ml of tap water was added. This water was replaced after 2 hours by 40 ml of distilled water, and flasks were then placed in a 24° incubator. Single lots of distilled and tap water were used throughout the study to avoid possible variation in the water samples. The number of zoospores at 15–17 hours was computed by counting 7 fields of a sample from each of 4 flasks in a standard haemacytometer. To stop the movement of zoospores, cellulose gum (Type 120 High, Hercules Powder Co.) was used at the rate of 0.3 g per 40 ml of zoospore suspension.

The 4 media used were: 1) pea decoction (per liter: extract from 41 g of Miragreen pea seeds), 2) maize decoction (per liter: extract from 41 g of maize kernels, field corn), 3) maltose-peptone broth, 4) potato-dextrose broth (per liter: extract from 200 g of potatoes; 10 g dextrose).

In an experiment on light effects, mycelial mats in distilled replacement water in open 300 ml preparation dishes were exposed to 150 ft-c from a 14-watt fluorescent lamp in an incubator at 24°.

In aeration studies, filtered air was introduced into distilled replacement water through 3-mm glass tubing at the rate of 300–400 ml per minute (ca. 6 bubbles per second). In one experiment the same amount of air was discharged at the surface of the water. Vacuum was applied by placing flasks in a desiccator

Table 1.—Zoospore production by *Aphanomyces euteiches* isolate 50 with different combinations of water changes.[a]

Test	Number of zoospores per ml (10,000's)						
	T-D	T-T	D-T	D-D	D	T	LSD 5%
1	18.6	4.9	14.1	9.6	10.7	1.7	7.2
2	20.0	3.2	13.3	14.5	18.7	1.4	5.8

[a] Mycelial washes indicated in sequence. T = tap water; D = distilled water.

connected to a water pump, and reducing pressure to 8 mm of mercury.

In all the experiments 4 replications per treatment were used. Analysis of variance was applied to the data, and statistically significant results are indicated for either the 5% or 1% level.

Results.—*Media.*—Zoospore production was compared using cultures grown in pea decoction, maize decoction, maltose-peptone broth, and potato-dextrose broth. Cultures of isolate 102 grown in all media sporulated poorly or not at all; a maximum of 7×10^4 zoospores per ml was produced in one experiment by cultures grown in maize decoction. Isolates 50, 72, and 103 behaved similarly, but the results of the 4 tests differed. In test 1 maltose-peptone broth gave significantly largest numbers of zoospores, in test 2 pea decoction was superior, and in tests 3 and 4 maize decoction gave the highest numbers. Maltose-peptone broth, pea decoction, and corn decoction always gave satisfactory zoospore production, in the range of 1–2.5 $\times10^5$ per ml of distilled replacement water, whereas potato-dextrose broth was distinctly inferior, particularly in tests 3 and 4. Since maltose-peptone broth could be formulated more precisely than the decoctions, it was used in all subsequent experiments.

Water requirements.—Two experiments were designed to determine the optimum combination of tap water and distilled water changes for zoospore production. Cultures of isolate 50 grown for 5 days at 24° were used. Treatments included immersing the mycelium first in tap water for 2 hours, then in distilled water for 15 hours, or vice versa, tap water followed by tap or unchanged distilled water. Zoospore numbers were largest when tap water was followed by distilled water (Table 1). In test 1 this treatment gave significantly more zoospores than the other treatments except distilled water followed by tap water. In test 2, tap water followed by distilled water was equalled only by distilled water alone. The numbers of zoospores produced in distilled followed by tap water or distilled followed by distilled water, was approximately two-thirds that of the best method. One or 2 changes of tap water gave significantly fewer zoospores.

The length of time the mycelial mats should be immersed in tap water after pouring off the medium was determined in 2 trials. Mats were placed in tap water for 1 minute, 15 minutes, 1 hour, 2 hours, or 6 hours before replacing with distilled water for 15–17 hours. Results were almost identical in both tests. When the fungus was left in tap water for 1 or 15 minutes very few zoospores were obtained as compared with mycelium in tap water for 1, 2, or 6 hours (Fig. 1). Counts for these latter 3 treatments did not differ significantly. In addition readings for the 6-hour treatment made at 17 and 23 hours did not differ. Therefore, the initial immersion in tap water, for at least 1 hour, was necessary to obtain a large number of zoospores. Leaving the fungus indefinitely in tap water, however, inhibited zoospore production.

Time interval for production of zoospores.—To determine the time of zoospore discharge, counts were made at 2-hour intervals after mycelial mats were placed in the distilled replacement water. No zoospores were present until 6 hours had elapsed (Fig. 2), and numbers were maximum in 8–10 hours. No zoospores were formed after 10 hours.

Temperature.—Studies were made to determine the optimum temperature for zoospore production. Mycelial mats grown 5 days at 23–25° were induced to sporulate at temperatures of 16°, 20°, 24°, 28°, and 32°. In 9 tests the maximum number of zoospores from isolates 52, 72, and 103 was consistently produced at 24° (Fig. 3). Number of zoospores produced at the 5 temperatures by isolates 52, 72, and 103 differed statistically in nearly all instances. The optimum temperature was not clearly defined for isolate 102 because it produced very few zoospores at all temperatures tested.

The effect of culture (growth) temperature on zoospore production of isolate 50 at 24° was evaluated in 3 tests. Mycelial mats grown at 20°, 24°, and 28° produced largest numbers of zoospores (Fig. 4). When these 3 temperatures were compared no significant differences were found except in test 1, in which numbers were highest at 24°. The data for these 3 temperatures differed from those at 16° and 32° in each test. Thus the optimum growth temperature range for maximum zoospore production was 20–28°.

Dry weight of the mycelium increased with temperature, reaching a peak at 28°. At still higher temperature (32°), growth was retarded (Fig. 4).

Age of the culture and volume of distilled replacement water.—The influence of the age of culture and volume of distilled replacement water on zoospore production was studied simultaneously in 3 experiments. Isolate 50 of *A. euteiches* was induced to form zoospores at 3, 5, 7, 9, and 11 days in 2 experiments and at 2, 4, and 6 days in another. When 30 or 50 ml of distilled replacement water were used, 5-day-old cultures produced significantly higher concentrations of zoospores than cultures that were 3, 7, 9, or 11 days old (Fig. 5). As the age of the culture increased beyond 5 days, zoospore production decreased. When 20 ml of water was used, the maximum concentration of zoospores was obtained with 3-day-old cultures; this treatment gave concentrations higher than any other. Results were similar in experiments 2 and 3.

When total numbers of zoospores per flask were considered, the maximum number was produced with cultures 5 days old in 30 or 50 ml of replacement water (Fig. 6). Cultures 3 days old produced a total

Table 2.—Effect of aeration of distilled replacement water on zoospore production by *Aphanomyces euteiches* isolate 50.

	Number of zoospores per ml (10,000's)								
Test	Vacuum	Air bubble 24°	Air at surface 24°	Open flasks 24°	Closed flasks 24°	Pre-aerated water 6°	Pre-aerated water 24°	Open flasks 6°	LSD 5%
1	0.0	108.5	34.2	22.4	14.2				9.2
2	0.0	49.9		21.8		19.0	25.7	16.1	8.5

number of zoospores greater than 5-, 7-, 9-, or 11-day-old cultures only when placed in 20 ml of distilled water.

Vegetative growth increased steadily over the period of the experiment. Therefore, zoospore production was not correlated with weight of mycelium.

Aeration.—Bubbling air into the distilled replacement water containing the mycelium of isolate 50 gave zoospore concentrations 2-4 times as great as those produced in open flasks (Table 2). In 1 test more than a million zoospores per ml were produced. However, when cultures were aerated at the water surface the number of zoospores was increased only slightly over that of nonaerated cultures. Zoospore production was not reduced by stoppering flasks, but vacuum completely prevented zoospore formation. When distilled water at 6° or 24° was aerated for 5 hours and then used as replacement water at an air temperature of 24° no significant increase in the number of zoospores was observed.

The air bubble method was used to induce isolate 102 to sporulate in 3 experiments. In all tests, aerated 4–5-day-old cultures produced numbers of zoospores on the order of $1–2\times10^5$ per ml, 6–10 times the numbers produced in open flasks (Table 3).

Equally high concentrations of zoospores were produced by aerating several mycelial mats together in the same flask. Four mats in 160 ml of water was a convenient number to use.

Light.—Placing sporulating mycelial mats of isolate 50 under 150 ft-c of light from a fluorescent lamp did not increase or decrease the number of zoospores produced.

Discussion.—The preceding experiments demonstrate the influence of several variable factors on zoospore production by *A. euteiches*, and the importance of standardizing these variables at optimum levels to obtain consistently high concentrations of zoospores. By using the information on the production of zoospores of *A. euteiches* for greenhouse inoculations of pea seedlings, fewer cultures are required and the danger of wasting valuable plant material owing to failure to obtain adequate inoculum is practically eliminated.

Maltose-peptone broth was an excellent, simple, and reproducible medium for culturing *A. euteiches* for zoospore production. It gave zoospore numbers comparable to those produced by pea decoction (1, 6) and maize decoction (4, 5), media commonly used by other workers.

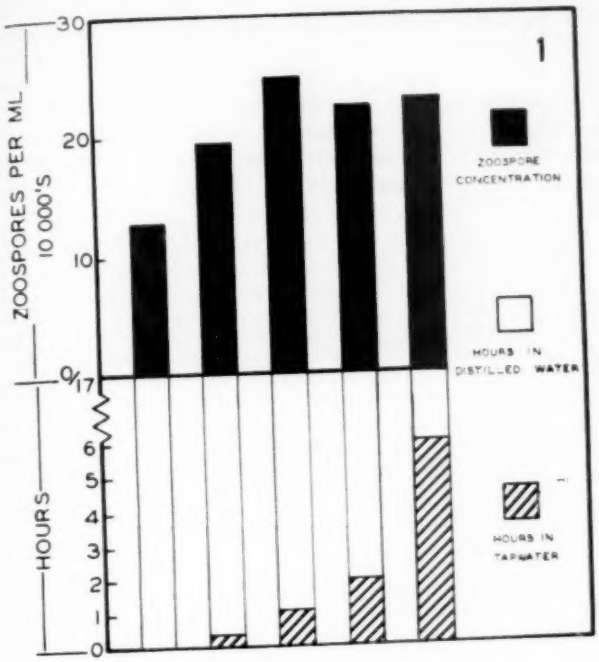
The method used to induce sporulation, placing mycelium in tap water followed by distilled water, was superior to several variations tried. In contrast with the results of others (5, 6), one or 2 changes of tap water gave very low numbers of zoospores. However, an initial immersion in tap water for at least 1 hour was necessary for maximum zoospore production. Differences in the tap water used would possibly account for the discrepancy. Zoospore discharge began in 5–6 hours, and was completed in 8–10 hours after the mycelial mats were placed in the distilled replacement water. These results are in general agreement with those obtained by other workers (1, 6).

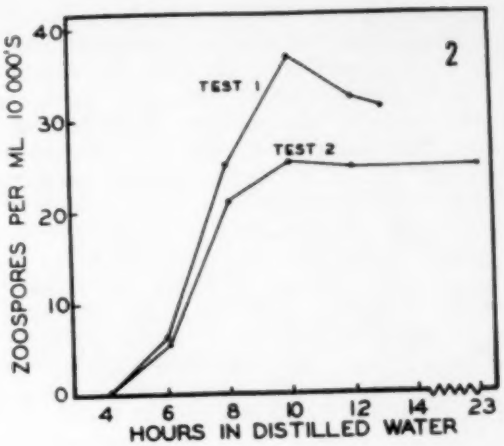
In conflicting reports on temperature optimum for zoospore production, Schneider and Johnson (4) gave the optimum range as 15–20°, whereas Sherwood (5) stated that numbers of zoospores were equal at a series of temperatures from 4° to 32°. In contrast with these reports, the optimum temperature for zoospore production for isolates 52, 72, and 103, in the present work in numerous experiments, was consistently 24°. Numbers were always substantially fewer at higher and lower temperatures.

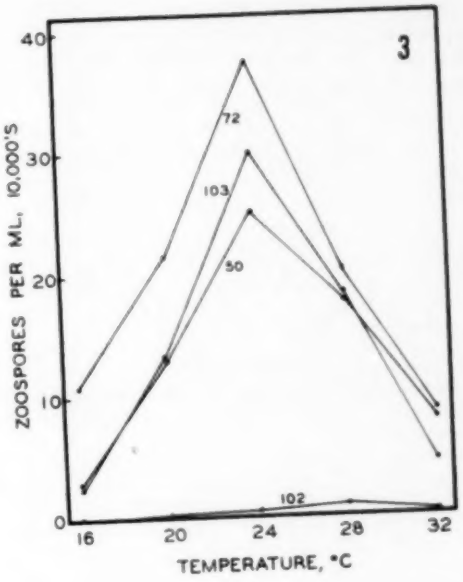
Table 3.—Effect of aeration of distilled replacement water on zoospore production by *Aphanomyces euteiches* isolate 102.

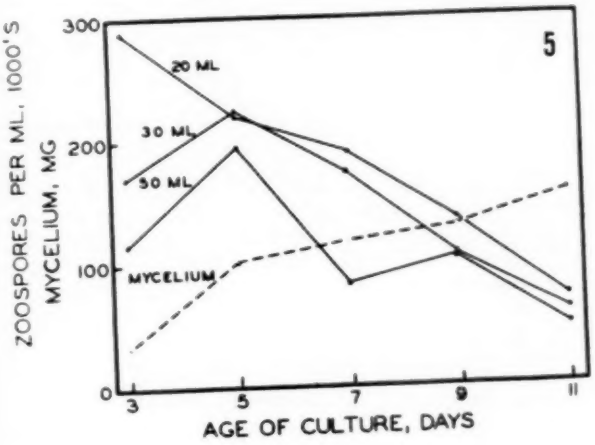
		Number of zoospores per ml (10,000's)	
Test	Age of culture	Air bubble	Open flasks
1	5 days	12.6	2.1
2	5 days	22.5	2.5
3	4 days	15.3	0.1
	10 days	1.0	0.0

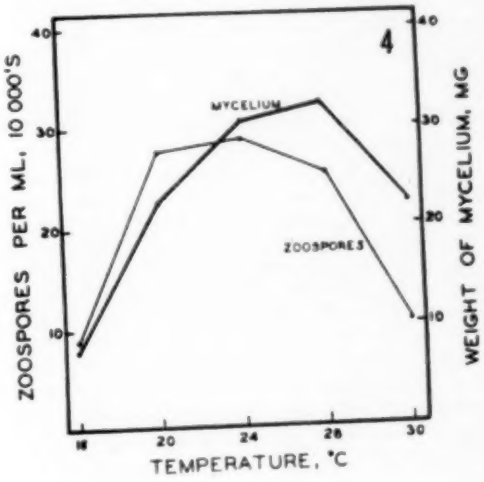
Fig. 1–6.—**1)** Zoospore production by *Aphanomyces euteiches* isolate 50 when mycelial mats were placed in tap water for varying periods before replacing with distilled water. LSD 5% = 8.2×10^4. **2)** Zoospore production by *Aphanomyces euteiches* isolate 50 at different time intervals when the mycelium was in distilled replacement water. LSD, 5%; test 1 = 5.6×10^4, test 2 = 11.9×10^4. **3)** Effect of temperature of distilled replacement water on zoospore production by isolates 50, 72, 102, and 103 of *Aphanomyces euteiches*. LSD 5% = 7.9×10^4. **4)** Effect of culture temperature of *Aphanomyces euteiches* isolate 50 on weight of mycelium and subsequent zoospore production. LSD 5% = 11.9×10^4. **5)** Effect of age of the culture and volume of distilled replacement water on concentrations of zoospores produced by *Aphanomyces euteiches* isolate 50. LSD 5% = 9.8×10^4. **6)** Effect of the age of the culture and volume of distilled replacement water on the total number of zoospores produced by *Aphanomyces euteiches* isolate 50.

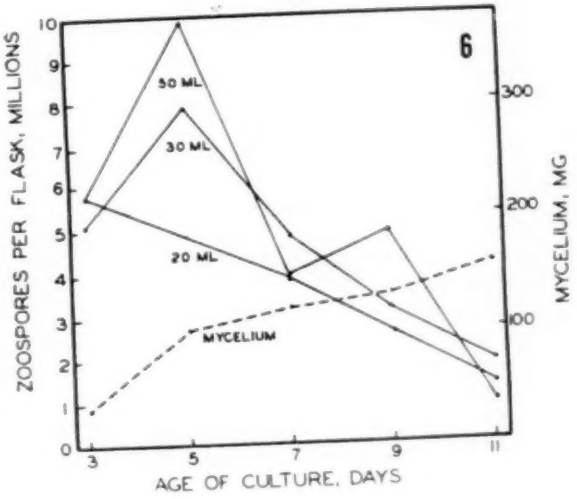

1
ZOOSPORES PER ML
10 000'S
HOURS
ZOOSPORE CONCENTRATION
HOURS IN DISTILLED WATER
HOURS IN TAPWATER


2
ZOOSPORES PER ML 10 000'S
TEST 1
TEST 2
HOURS IN DISTILLED WATER


3
ZOOSPORES PER ML, 10,000'S
72
103
50
102
TEMPERATURE, °C


5
ZOOSPORES PER ML, 1000'S
MYCELIUM, MG
20 ML
30 ML
50 ML
MYCELIUM
AGE OF CULTURE, DAYS


4
ZOOSPORES PER ML, 10 000'S
WEIGHT OF MYCELIUM, MG
MYCELIUM
ZOOSPORES
TEMPERATURE, °C


6
ZOOSPORES PER FLASK, MILLIONS
MYCELIUM, MG
50 ML
30 ML
20 ML
MYCELIUM
AGE OF CULTURE, DAYS

Vegetative growth of isolate 50 increased steadily with temperature, reaching its maximum at 28°, results agreeing with those of Smith and Walker (6). Maximum mycelial growth, however, was not necessarily associated with maximum production of zoospores. The vegetative growth of the organism was less at 20° than at 24° or 28°, but as many zoospores were produced by cultures grown at 20° as by those grown at the higher temperatures. Likewise, increase in weight of mycelium due to increasing age of cultures was not correlated with increased zoospore production. In general, cultures 5 days old produced more zoospores than younger or older cultures.

Zoospore production in *A. euteiches* was increased dramatically by aeration of the distilled replacement water, a response apparently not previously reported. Zoospore concentrations produced by isolate 50 were increased 2–4 times by such treatment. Isolate 102, which normally did not produce adequate numbers of zoospores for inoculation of plants, became an excellent zoospore producer when air was bubbled into the distilled replacement water. This method, which is used for routine production of zoospores for inoculation of peas at Michigan State University, reduces to ½ or ¼ the number of cultures required. Combining several mats in 1 flask for zoospore production gives further savings in equipment and handling.—Department of Botany and Plant Pathology, Michigan State University, East Lansing.

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MECHANISMS OF FUNGITOXIC ACTION OF n-DODECYLGUANIDINE ACETATE

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SUMMARY

In a homologous series of n-alkyl guanidine acetate compounds with alkyl groups of 10–18 carbons, maximum toxicity to cells of *Saccharomyces pastorianus* and conidia of *Monilinia fructicola* was exhibited by the homologs with alkyl groups of 13–14 carbons. Maximum phytotoxicity to *Nicotiana glutinosa* was exhibited by the homolog with an alkyl group of 10 carbons. Toxicity of dodine to *S. pastorianus* increased as pH of the external medium was increased from 5.1 to 7.8. Dodine was rapidly accumulated in large quantities by fungus cells. Inhibition, by dodine, of the oxidation of glucose by cells of *S. pastorianus* was closely correlated with inhibition of growth of this organism. The effect of dodine on the oxidation of glucose by conidia of *M. fructicola* was not correlated with the effect of this compound on spore germination. Fermentation of glucose by intact yeast cells or of glucose and fructose-1,6-diphosphate (HDP) by cell-free extracts of the yeast was strongly inhibited by dodine. Under anaerobic conditions, pyruvate was not fermented by untreated yeast cells, but cells pretreated with dodine readily fermented this substrate. Cell-free extracts of the yeast metabolized pyruvate regardless of whether the extracts were prepared from untreated cells or from cells pretreated with dodine, although activity was less in the latter. Exogenous sources of HDP were not metabolized by untreated yeast cells, as indicated by aldolase activity, but this substrate was metabolized by cells pretreated with dodine. Cells of *S. pastorianus* and conidia of *M. fructicola* lost phosphorus compounds and ninhydrin-positive substances when treated with toxic doses of dodine. The loss of cellular constituents was considerably more prominent in the former. Alteration of permeability of the cell with the resulting loss of vital cellular constituents, and inactivation of certain vital enzymes, appear to be the mechanisms by which dodine exerts its toxic effect on *S. pastorianus*. The toxicity of dodine to conidia of *M. fructicola* is attributed to the blocking of vital anionic sites at the cell surface and to inactivation of certain vital enzymes.

The fungicidal compound n-dodecylguanidine acetate, commonly referred to as dodine or Cyprex, has recently achieved prominence as a fungicide for the control of certain plant diseases. The toxicant is a cationic surfactant and has the structural formula:

$$C_{12}H_{25}—NH—\overset{\overset{NH}{\|}}{C}—NH_2 \cdot CH_3COOH$$

It consists of a straight-chained alkyl group of 12 carbons attached to the strong base guanidine, which is neutralized by acetic acid.

The present investigation was undertaken to determine the manner in which dodine exerts its toxic effect on fungal cells. There is no literature concerning the mechanism of action of this particular compound, but some information is available on the mode of action of other cationic surface-active toxicants.

West and Wolf (19) presented data indicating that the fungicidal compound 2-heptadecyl-2-imidazoline interferes with purine metabolism in *Monilinia fructicola*. Hotchkiss (7) showed that cationic surfactants cause bacterial and yeast cells to lose nitrogen- and phosphorus-containing compounds to the external medium and suggested that the loss of these substances resulted from cytolytic damage to the cells. Gale and Taylor (5) detected the loss of amino acids from cells of streptococci in the presence of bactericidal dosages of surfactants. Such cells failed to oxidize glucose, pyruvate, L-alanine, or succinate.

Inhibition of enzyme activity by cationic surface active agents has been reported in several instances. Knox et al (9) found that 50% inhibition of glucose oxidation and of growth was achieved in cells of *E. coli*, by similar dosages of the cationic surfactant Zephiran. Inhibition of enzymes, such as catalase and arginine decarboxylase, however, required considerably higher dosages of the toxicant. Activity of the latter enzyme was arrested only in the presence of sufficient surfactant to bring about denaturation of the apoenzyme. Other data of these workers show that glycolytic enzymes are sensitive to this cationic surfactant in the intact cell, but resistant when isolated.

Materials and methods.—*Cultural procedures.*—The organisms used in this study were *Saccharomyces pastorianus* Hans. and *Monilinia fructicola* (Wint) Honey. Stock cultures of the former organism were maintained at 4°C, and small quantities transferred to a nutrient medium for the purpose of obtaining inoculum. The nutrient medium consisted of the following: glucose, 10 g; KH_2PO_4, 2.0 g; K_2HPO_4, 1.5 g; $(NH_4)_2SO_4$, 1.0 g; $MgSO_4$, 0.5 g; trace elements consisting of 0.05 ppm each of manganese as $MnSO_4$, zinc as $ZnSO_4$, copper as $CuSO_4$, iron as $FeSO_4$, and molybdenum as Na_2MoO_4; vitamins consisting of thiamine hydrochloride, 200 μg; calcium panthethenate, 200 μg; niacin, 200 μg; biotin 10 μg; i-inositol, 2.0 mg; pyridoxine, 100 μg; and distilled water to 1 L. The pH of the medium was 6.4. The culture flasks were placed on a reciprocating shaker and the yeast grown at room temperature for about 12 hours. The suspension was then centrifuged, and the cells were washed twice and then suspended in the nutrient medium or distilled water. It was necessary to use distilled water in some instances because high concentrations of dodine formed a precipitate in the presence of inorganic salts. Cell suspensions were adjusted to an optical density reading of 0.70 or 1.0 at 450 mμ. These readings respectively correspond to 0.43 and 0.65 mg dry wt./ml. A Coleman Spectrophotometer was used for this purpose.

Monilinia fructicola was cultured in Petri plates on Difco potato-dextrose agar. The culture plates were inoculated with a sterile suspension of conidia and maintained at 25°C. Conidia were harvested at 7 days by flooding the cultures with distilled water and gently rubbing the surface of the culture with a rubber policeman. The resulting suspension was filtered through cheesecloth to remove mycelial fragments. The filtrate was centrifuged, and the conidia were washed and then suspended in a volume of distilled water to give an optical density of 1.0 at 450 mμ. This optical density corresponds to a dry weight of 0.75 mg/ml.

Evaluation of the toxicity of dodine.—The toxicity of dodine to cells of *S. pastorianus* was determined by suspending cells of the yeast in the nutrient medium in the presence of the fungicide. Growth of the test organism was determined by the optical density of the cultures. Specifically, 9.4 ml of the nutrient medium was pipetted into screw-cap test tubes (125×20 mm) that would fit into the spectrophotometer. One-half ml of yeast inoculum (0.43 mg dry wt./ml) and 0.1 ml of the desired concentration of dodine in 95% ethanol were added to the medium. The initial optical density of the cultures was 0.04. When cultures used as controls reached an optical density of about 1.0, the test was terminated. This method was used in studying the effect of the hydrogen-ion concentration of the external medium on the toxicity of dodine and in evaluating the relative toxicity of a homologous series of alkyl guanidines.

In most experiments more concentrated suspensions (0.65 mg dry wt./ml) were used. The effective dosage (ED) values of dodine at this level of cells were obtained by an agar plating method. Cells were treated with the desired concentrations of dodine in distilled water for 15 minutes, a 0.04-ml aliquot was added to 100 ml of distilled water and mixed well, and 0.05 ml was spread evenly on the surface of an agar medium. This was the same nutrient medium as described previously to which 3 g/L of bacto agar was added. After 36 hours, the yeast colonies were counted.

In manometric and other types of experiments in which this quantity of yeast was used, the cells were treated with the toxicant for 15 minutes and sedimented, and the supernatant solution decanted. The cells were washed once with distilled water and the suspension then brought up to the original volume with the desired medium and used directly. The pH of the distilled-water solutions of dodine was 6.4–6.5.

Inhibition of spore germination was used as a measure of the toxicity of dodine to *M. fructicola*. A spore was considered germinated when the germ tube reached a length of about one-half the width of the spore. Conidia of the fungus were suspended in 4.95 ml of distilled water to which 0.05 ml of the appropriate concentration of dodine was added. After shaking for 15 minutes, 0.5 ml of the treated conidial suspension was placed in a Petri plate containing 8 ml of ¼-strength Difco potato-dextrose agar medium to which 1 g/L of bacto agar had been added. At least 90–95% of the conidia used as a control germinated in 4 hours at 25°C on this medium. This particular method was also used to determine the relative toxicity of the homologous series of alkyl guanidines to *M. fructicola*. In manometric experiments, samples of the conidial suspensions were removed from vessels at the termination of the gas measurements and per cent germination was determined by standard slide germination techniques after a 1–3-hour incubation period at room temperature.

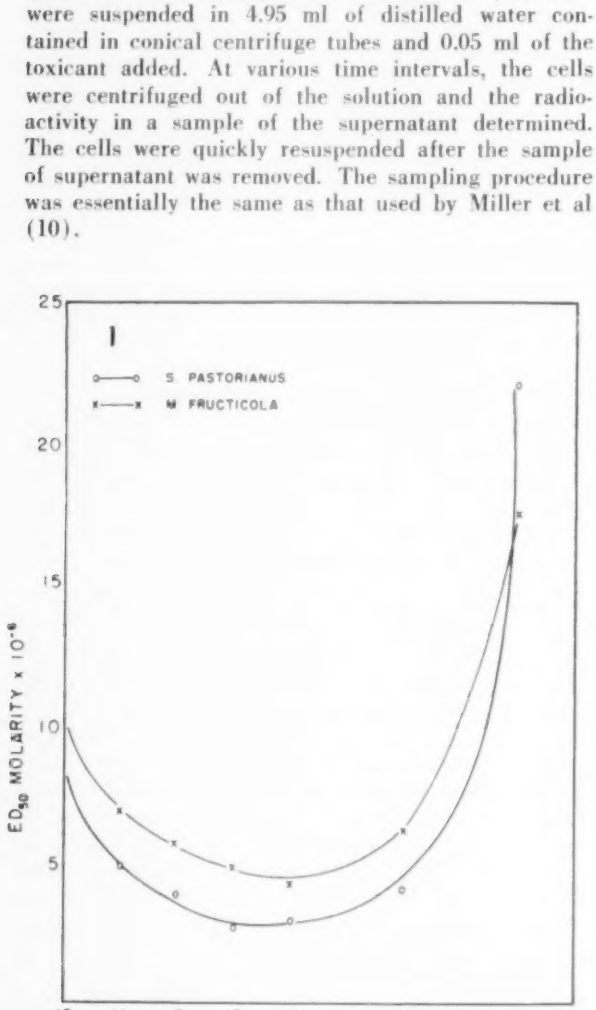
Radioactive isotopes.—^{14}C-labeled dodine and ^{32}P-labeled phosphate were used in several experiments. Radioactivity of the ^{14}C-labeled fungicide was measured with a Tracerlab SC-16 windowless gas-flow counter, and ^{32}P activity was determined with an end-window Geiger-Muller tube. These apparatuses were attached to a SC-1C autoscaler equipped with a preset counter. The radioactive solution was prepared for counting by placing a 0.1-ml aliquot into a metal planchett and drying it under a 150-W lamp.

Uptake of ^{14}C-labeled dodine.—The uptake of dodine by the test organisms was determined by using ^{14}C-labeled dodine. Nonradioactive dodine was added to aliquots of a known uniform amount of the radioactive toxicant so that the final concentration of three test dosages was 5.62, 10.0, and 56.2 ppm. In this manner, the initial count per minute of each dosage was held constant. Cells of the yeast or conidia of *M. fructicola* were suspended in 4.95 ml of distilled water contained in conical centrifuge tubes and 0.05 ml of the toxicant added. At various time intervals, the cells were centrifuged out of the solution and the radioactivity in a sample of the supernatant determined. The cells were quickly resuspended after the sample of supernatant was removed. The sampling procedure was essentially the same as that used by Miller et al (10).

The ^{14}C-labeled dodine and the homologous series of n-alkyl guanidines were kindly supplied by the American Cyanamid Company.

Loss of constituents from cells in the presence of dodine.—Radioactive phosphate was used to determine the loss of phosphorus-containing substances from the cells. The yeast was grown in a low phosphate ($2.2\times10^{-4}M$) nutrient medium to facilitate the accumulation of ^{32}P-labeled phosphate in subsequent experiments. The phosphorus-deficient cells were washed and a known quantity was suspended in nutrient medium minus unlabeled phosphate but containing a small quantity of ^{32}P-labeled phosphate and $0.1M$ citrate buffer adjusted to pH 6.4 with sodium hydroxide. Potassium ions were supplied as potassium chloride. After 60 minutes, the cells were centrifuged out of the radioactive medium, washed, and returned to the original volume with distilled water. Volumes of 4.95 ml were then treated with dodine in the manner previously described. Conidia of *M. fructicola* (0.75 mg dry wt./ml) were handled in the same manner, but with the following exceptions: The conidia were allowed to take up ^{32}P-labeled phosphate for 120 minutes with no previous growth in the low-phosphate medium. The procedure for sampling the supernatant solution at various time intervals was the same as that used in measuring uptake of labeled dodine.

In determining the type of phosphorus compounds lost by cells and in determining the loss of ninhydrin-positive compounds, large volumes of the test organisms were treated with various concentrations of dodine for 15 minutes. The cells were sedimented and the supernatant condensed to 3 ml *in vacuo*. Control samples were handled in a similar manner.



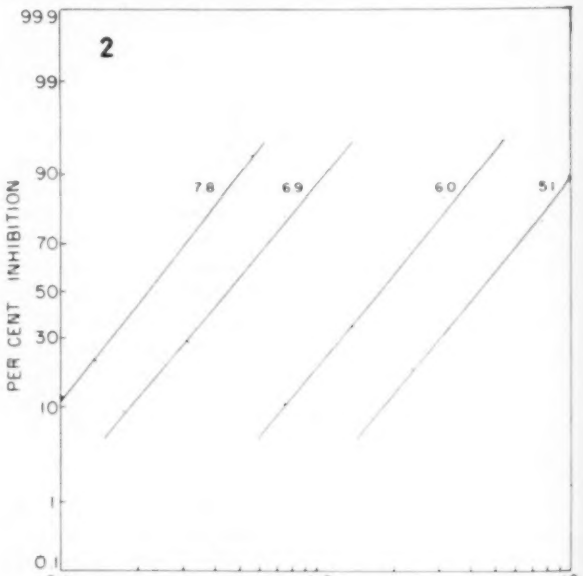


Fig. 1–2.—**1)** The relative toxicity of members in a homologous series of n-alkyl guanidine acetate compounds to *Monilinia fructicola* and *Saccharomyces pastorianus*. **2)** The effect of hydrogen-ion concentrations of pH 7.8, 6.9, 6.0, and 5.1 on the toxicity of dodine to cells of *Saccharomyces pastorianus*. Toxicity values are plotted on logarithmic probability paper.

Total ninhydrin-positive substances lost to the medium by the cells were determined by the method of Troll and Cannan (16). These compounds were separated two-dimensionally on Whatman No. 1 filter paper sheets using water-saturated phenol in the first dimension and n-butanol–acetic-acid–water, 250:60:250 v/v/v, in the second dimension. The chromatograms were air-dried and sprayed with 0.25% ninhydrin in acetone and heated for 10 minutes in an oven at 85°C.

Separation of the phosphorylated compounds was accomplished on Whatman No. 3 filter-paper sheets with a solvent system of isobutyric-acid–ammonium-hydroxide–water, 57:4:39 v/v/v, slightly modified from Kilgour et al (8). The solvent was used ascending for 8 hours, the paper inverted 90°, and run descending until the solvent front reached the edge of the chromatogram. The chromatograms were dried and placed next to X-ray film in exposure holders. After several days of exposure, depending on the activity present, the films were removed and developed.

The identity or group to which the phosphorus compounds belonged was determined by comparing R_f values of spots on the radioautograms with the R_f values of known phosphorus compounds and by using ultraviolet light. The unlabeled phosphorus compounds were located on the chromatograms by the method of Hanes and Isherwood (6).

Inhibition of phosphorus uptake.—Cells of the test organisms were pretreated for 15 minutes in distilled water with various concentrations of dodine and then placed in the nutrient medium minus labeled phosphate but containing a small quantity of ^{32}P-labeled phosphate. At the end of various time intervals, samples of the solution were removed for radioactivity measurements. The procedure for sampling was the same as that used in measuring the uptake of dodine.

Manometry.—Conventional Warburg manometric techniques were used to study respiration and fermentation. The water-bath temperature was maintained at 30°C for *S. pastorianus* and 25°C for *M. fructicola*. The shaking rate was 120 cycles/minute.

Respiration.—Cells of *S. pastorianus* and conidia of *M. fructicola* were pretreated with dodine in the usual manner and washed. The yeast cells were suspended in nutrient medium minus the nitrogen source, and the conidia were suspended in a 0.022*M* phosphate buffer solution (pH 6.4) containing 1.0% glucose. Two ml of the suspending medium were placed in the main compartment of the vessel, and 0.2 ml of 2% KOH was added to the center well.

Fermentation.—Intact cells of *S. pastorianus* as well as cell-free extracts from this organism were used in studies of fermentation in an atmosphere of nitrogen. Substrates used were glucose and the sodium salts of fructose-1.6-diphosphate, and pyruvate, each at a final concentration of 0.1*M* in a 0.1*M* citrate buffer of pH 6.4. A solution of the following cofactors was added so that each vessel contained diphosphopyridine nucleotide (DPN), 1.0 mg; adenosine, 2.0 mg; acetaldehyde, 1.0 mg; thiamine pyrophosphate, 0.15 mg; adenosine triphosphate (ATP), 1.0 mg; phosphate as potassium phosphate, 2 mg; and magnesium as $MgSO_4$, 1 mg. Magnesium and thiamine pyrophosphate only, were added at the same levels when pyruvate was the substrate. The final fluid volume in the vessels was 2 ml.

Correction was made for CO_2 retention in the medium according to the method described by Umbreit et al (17).

In preparing cell-free extracts, 300 ml of yeast cells (0.65 mg dry wt./ml) were treated for 15 minutes with 31.6 ppm of dodine or left untreated in the case of the control samples. The cell suspension was centrifuged and the cells washed twice with distilled water and brought up to a final volume of 40 ml in a 0.1*M* citrate buffer of pH 6.4. This volume of cells was exposed to sonic waves in a Raytheon Sonic Disintegrator model DF-101 in the presence of 1.0 g of celite. Over 95% of the cells were broken in an exposure period of 15 minutes. The holding cup of the apparatus was maintained at 5°C during the breaking period. The broken-cell preparation was centrifuged at 2000 rpm for 10 minutes in a Servall refrigerated centrifuge at 5°C to remove cell walls and other large fragments. One ml of the cell-free extract was used in each Warburg vessel.

Aldolase activity.—The effect of dodine on the utilization of glucose-6-phosphate and fructose-1, 6-diphosphate (HDP) by cell-free extracts of *S. pastorianus* was determined by measuring aldolase activity. The method of Sibley and Lehninger (14) was followed except for the use of a 0.1*M* citrate buffer adjusted to pH 6.4 instead of Tris buffer of pH 8.6, and a water-bath temperature of 30°C instead of 38°C. One-half ml of the cell-free extract was used as a source of enzymes. Glucose-6-phosphate and HDP were used as the sodium salts at 0.1*M*. With the former substrate, ATP was used at the same rate as in the manometric experiments. Aldolase activity was also determined in this manner when these substrates were added to intact cells. In these experiments, 40 ml of cells (0.65 mg dry wt./ml) were pretreated with 31.6 ppm of dodine, centrifuged, washed twice, and brought up to a volume of 10 ml with 0.1*M* citrate buffer. One-half ml of these cell suspensions was used in each tube as a source of enzymes. Control cell suspensions not treated with dodine were prepared in a similar manner.

Phytotoxicity of n-alkyl guanidines.—The phytotoxicity of a homologous series of n-alkyl guanidines was determined by placing 0.01-ml aliquots of the toxicants on the leaves of *Nicotiana glutinosa* (glutinosa) and *Phaseolus vulgaris* var. Pinto. Quantities of each homolog were prepared by diluting a stock solution of 80,000 ppm in 95% ethanol with distilled water to give final concentrations of 125, 250, 500, 1000, and 2000 ppm. Control applications consisted of the same series of dilutions of 95% ethanol. Four leaves on each of eight glutinosa plants were chosen, and each half leaf was marked with India ink to designate the location at which the toxicant would be applied. In this manner, the controls and the 7 homologs

Table 1.—Relative toxicity of a homologous series of n-alkyl guanidines to leaves of *Nicotiana glutinosa*.

Dosage (ppm)	Phytotoxic values[a] Number of carbons in alkyl group						
	10	11	12	13	14	16	18
125	0.45	1.4	0.85	0.70	0.33	0	0
250	6.83	4.57	5.86	2.87	0.50	0	0
500	10.88	11.25	7.75	5.00	1.25	0	0
1,000	15.13	11.78	5.20	3.87	0.95	0	0
2,000	23.10	15.50	5.73	4.60	1.00	0	0

[a] Injury was rated with a scale of 0–3. A value of 3 indicates severe injury; 2, moderate; and 1, slight injury. Values in table are the sum of the injury values of 8 replicates of each treatment. Maximum injury value possible is 24.

at all dosages appeared once on every plant. A similar technique was followed in applying the toxicants to the first true leaves of pinto bean. Three days after application, the degree of phytotoxicity was evaluated using a numerical system of 0–3, from no injury to severe damage.

Results.—*Relative fungitoxicity and phytotoxicity of n-alkyl guanidines.*—It has been shown in a number of instances that the biological activity of surface-active agents within a homologous series is dependent on the length of the hydrocarbon chain (13). The optimum chain length for maximum activity, however, is not the same for all organisms. Wellman and McCallan (18), for instance, demonstrated that the optimum chain length for fungitoxicity in a homologous series of n-alkyl imidazolines is not the same as that producing maximum phytotoxicity. Similar observations were made by Ross and Ludwig (12) for a homologous series of N-n-alkylethylenethioureas. When certain barriers such as seed coats and cell walls of the higher plant cells were removed, maximum fungitoxicity and phytotoxicity in this series occurred at the same chain length.

The influence of the hydrocarbon chain length on the fungitoxicity and phytotoxicity in a homologous series of n-alkyl guanidine acetate compounds was investigated. Available for this purpose were guanidine compounds that contained alkyl groups of 10, 11, 12 (dodine), 13, 14, 16, and 18 carbons.

In fungitoxic tests there were only small differences in the effectiveness of homologs with alkyl groups of 11–16 carbons (Fig. 1). The C_{13} homolog exhibited greatest toxicity toward *S. pastorianus*, whereas the C_{14} homolog was most toxic to *M. fructicola*. Toxicity drops off sharply with the C_{10} and C_{18} members of the series. It appears that members of this series with alkyl groups of less than 10 carbons or more than 18 carbons would possess low fungitoxicity. Guanidine acetate itself was not toxic to the yeast cells, even at 2000 ppm.

Since the amount of inoculum used in the case of *S. pastorianus* (0.2 mg dry wt/ml) differs widely from that used in the case of *M. fructicola* (0.75 mg dry wt/ml) no comparisons can be made between the relative toxicity of the homologs to the two organisms. In tests with comparable amounts of inoculum, *M. fructicola* was more sensitive to dodine than *S. pastorianus*.

Studies of the phytotoxicity of the homologs showed that the leaves of *N. glutinosa* and Pinto bean differed markedly in sensitivity to the toxicants. No injury was caused by any homolog applied to leaves of the bean plants in concentrations up to 2000 ppm. When the same concentrations were placed on glutinosa leaves, however, severe injury was caused by the higher concentrations of certain members of the series (Table 1). It is apparent that phytotoxicity increases as the homologous series is descended, with maximum toxicity occurring at the C_{10} homolog. Thus, in this series of alkyl guanidines, the optimum chain length for fungitoxicity differs from that producing maximum phytotoxicity.

Judging from the results of these experiments it would appear that homologs other than dodine might warrant further investigation as fungicides for the control of plant diseases. There is an indication that the C_{14} and C_{16} homologs might afford protection against pathogenic fungi equal to or greater than that provided by dodine, but with less phytotoxicity. These homologs might be useful in the control of plant diseases in cases where phytotoxicity with dodine has been encountered.

Effect of the hydrogen-ion concentration on toxicity.—The hydrogen-ion concentration of the external medium often affects the toxicity of ionic surfactants. Baker et al (1), for instance, demonstrated that the toxicity of cationic surfactants to *Streptomyces aureus* and to *E. coli* is enhanced by increased alkalinity of the external medium.

The toxicity of dodine to *S. pastorianus* is also affected by the hydrogen-ion concentration of the external medium. The respective concentrations of dodine necessary to inhibit 50% of the growth of the yeast at pH levels of 7.8, 6.9, 6.0, and 5.1 were 0.21, 0.42, 1.78, and 4.2 ppm (Fig. 2). Thus, a 20-fold increase in the dosage of dodine is required to maintain a constant degree of inhibition of growth when pH of the external medium is lowered from 7.8 to 5.1.

In this case it is unlikely that these changes in the hydrogen-ion concentration are influencing the toxicity of dodine by altering the charge of the guanidino group. Guanidino groups such as found in arginine, for instance, are strongly basic, with pK's in the vicinity of 12.5 (4). Thus, over the pH range of these tests, the guanidino group of dodine would be fully ionized. It is considered most likely that the hydrogen-ion concentration influences toxicity in this case by affecting the magnitude of ionization of anionic sites such as carboxyl and phosphate groups of the cell surface to which dodine attaches. The pK's of these groups are such that the degree to which they are ionized would vary with hydrogen-ion concentration within the pH range used in these experiments.

In all experiments that follow, *S. pastorianus* was used at a concentration of 0.65 mg dry wt/ml instead of 0.02 mg dry wt/ml, but *M. fructicola* was used at

the same concentration as in the previous experiments. The ED_{50} value of dodine for the higher level of yeast is 6.02 ppm at pH 6.4 and 2.50 ppm for *M. fructicola* at the same pH.

Uptake of dodine.—Miller et al (10, 11) have shown that spores of some fungi are capable of rapidly accumulating large amounts of toxic compounds from dilute solutions. A study of the uptake of ^{14}C-labeled dodine by yeast cells and by spores of *M. fructicola* showed that these organisms also accumulate large quantities of the toxicant very rapidly. Eighty-five to ninety per cent of the dodine ultimately taken up by the yeast cells was removed from the external medium during the first minute. Uptake by *M. fructicola* was somewhat less rapid, especially at the concentrations of 10 and 56.2 ppm, but the quantities taken up at the end of 3 minutes compared favorably with those taken up by yeast cells in the same period (Table 2).

A feature characteristic of cationic surface-active agents is their tendency to adsorb to glass. About 33% of the dodine added to culture tubes was lost to the glass walls in the first minute in the absence of fungus cells. When the toxicant was added to culture tubes that contained fungus cells, however, the amount lost to glass was 20% of the quantity available. The adsorption to glass in the latter case was determined after removing the toxicant from the glass with 95% ethanol. Essentially all adsorption of dodine occurred in the first minute. Values in Table 2 are corrected for losses to glass.

Effect of dodine on respiration.—The effect of dodine on the ability of cells of *S. pastorianus* and of conidia of *M. fructicola* to oxidize glucose revealed interesting differences between the two organisms. In the case of *S. pastorianus*, the inhibition of oxygen uptake is closely correlated with inhibition of growth (Fig. 3). Germination of conidia of *M. fructicola*, however, was considerably more sensitive to dodine than the oxidation of glucose by these cells. A 3.16-ppm concentration of the fungicide inhibited sport germination by 54%, but actually stimulated the rate of oxygen uptake (Fig. 4). Even when germination was almost completely inhibited, the oxidation of glucose was reduced only 67%.

There was no appreciable difference in the oxygen uptake by either organism when dodine was allowed to remain in solution with the cells or when the cells were pretreated with the toxicant and then washed before respiration measurements were made.

The oxidation of pyruvate by the yeast cells was completely inhibited by 31.6 ppm of dodine, indicating an inhibition of enzymatic activity at some point in the aerobic portion of the respiratory system.

Fermentation.—The fermentation of glucose by cells of *S. pastorianus* was strongly inhibited when they were pretreated with 31.6 ppm (ED_{98}) of the toxicant and washed before exposure to the substrate (Fig. 5). Inhibition was as marked when the cells were treated with this concentration of the toxicant and exposed to substrate without washing.

Cell-free extracts from untreated cells of the yeast fermented glucose and HDP readily, but the fermentation of these substrates was strongly inhibited in extracts from cells treated with 31.6 ppm of dodine (Fig. 6). Thus it is apparent from these studies of fermentation by whole cells and by cell-free extracts that toxic concentrations of dodine affect one or more enzymes in the fermentative system of this yeast.

The interesting observation was made that pyruvate under anaerobic conditions is metabolized much more rapidly by whole cells treated with 31.6 ppm of dodine than by untreated cells (Fig. 5). On the other hand, metabolism of pyruvate was more rapid in cell-free extracts from untreated cells than in extracts from cells treated with dodine (Fig. 6).

It has been observed by others (3) that pyruvate ions are rapidly metabolized by yeast cells in the presence of oxygen but not under anaerobic conditions. Undissociated pyruvic acid is metabolized under both anaerobic and aerobic conditions. The failure of yeast cells to metabolize pyruvate ions under anaerobic conditions is attributed to the lowering of cell permeability under nitrogen (15).

The results of the present experiments are in agreement with the observations that yeast cells do not normally metabolize pyruvate under anaerobic conditions except that in *S. pastorianus* there is a weak metabolism of this ion. Since cells treated with dodine readily metabolize pyruvate under anaerobic conditions their permeability to this ion has apparently been altered.

Aldolase activity.—The inhibitory effect of dodine on the enzyme aldolase and other enzymes occurring prior to aldolase in the fermentative pathway was studied by determining aldolase activity in cell-free ex-

Table 2.—Uptake of ^{14}C-labeled dodine by *Saccharomyces pastorianus* and *Monilinia fructicola*.

Organism	Dodine (ppm)	Dodine available (μg/g fresh weight)	Cumulative uptake of dodine μg/g fresh weight				
			Time (minutes)				
			1	3	5	10	15
S. pastorianus	5.62	2,611	1,650	1,890	1,930	1,940	1,940
	10.0	4,629	3,240	3,390	3,460	3,470	3,560
	56.2	26,111	16,000	17,700	18,600	18,400	18,300
M. fructicola	5.62	2,611	1,540	1,920	1,990	1,990	2,010
	10.0	4,629	1,720	2,890	2,970	3,270	3,390
	56.2	26,111	10,300	16,200	18,400	19,300	19,500

Table 3.—The effect of 31.6 ppm of dodine on the utilization of various substrates by intact cells and cell-free extracts of *Saccharomyces pastorianus* as indicated by aldolase activity.[a]

Substrate		Optical density at various intervals (minutes): 10	20	30	40	50
Intact cells						
Fructose-1,6-diphosphate	Control	0.01	0.01	0.03	0.04	0.05
	Treated	0.06	0.20	0.31	0.43	0.56
Glucose-6-phosphate	Control	0.0	0.0	0.0	0.0	0.0
	Treated	0.0	0.04	0.02	0.03	0.03
Cell-free extract						
Fructose-1,6-diphosphate	Control	0.07	0.25	0.51	0.75	0.95
	Treated	0.04	0.12	0.18	0.26	0.32
		Intervals (minutes) 3	6	9	12	15
Glucose-6-phosphate	Control	0.06	0.12	0.22	0.48	0.76
	Treated	0.05	0.07	0.06	0.07	0.08

[a] The activity was determined colorimetrically by measuring the optical density of the 2,4-dinitrophenyl hydrazine derivatives of the trioses under alkaline conditions.

tracts. When HDP was the substrate, aldolase activity was inhibited 66% in extracts prepared from cells pretreated with 31.6 ppm of dodine (Table 3). With a substrate of glucose-6-phosphate, however, there was practically no aldolase activity in extracts from cells treated with dodine, but aldolase activity in extracts from untreated cells was nearly as great as when HDP was the substrate.

Thus it appears that either phosphoglucoisomerase or 6-phosphofructokinase or both are strongly inhibited by dodine. The failure of cell-free extracts to ferment HDP also indicate that one or more enzymes between aldolase and carboxylase in the fermentative system are more strongly inhibited by dodine than is aldolase.

It is well known that intact cells of yeast do not metabolize exogenous sources of phosphorylated sugars such as HDP and glucose-6-phosphate, even though these compounds are intermediates of glucose metabolism. Permeability restrictions of the cell probably prevent the utilization of these substrates. Since dodine apparently alters the permeability of the yeast cells sufficiently to allow them to ferment pyruvate, the possibility was considered that such cells might also be made permeable to phosphorylated sugars.

The data in Table 3 show that cells pretreated with dodine are capable of utilizing HDP whereas untreated cells are inactive in this respect. Neither treated nor untreated cells would utilize glucose-6-phosphate, as indicated by aldolase activity. This observation is in accord with the results obtained with cell-free extracts where glucose-6-phosphate is not used by extracts from treated cells because of strong inhibition of enzymes occurring prior to aldolase in the fermentative system. It therefore seems most likely that glucose-6-phosphate passed the permeability barrier in the cells treated with dodine, but was not metabolized, because the required enzymes were inactivated.

Loss of cellular constituents.—The fact that cells of *S. pastorianus* metabolized HDP and fermented pyruvate only after they were treated with dodine suggested that alteration of cell permeability may be an important feature in the mechanism of action of this fungicide. Further experiments of a different nature were made to determine the extent to which dodine alters permeability of fungus cells.

Cells of *S. pastorianus* and conidia of *M. fructicola* lose phosphorus compounds and ninhydrin-positive substances when treated with toxic doses of dodine (Tables 4, 5). Loss of cellular constituents is much more prominent in *S. pastorianus* than in *M. fructicola*. In the presence of 56.2 ppm of dodine (ED_{99}), cells of the former organism lost 65% of the ^{32}P previously taken up. The loss of ninhydrin-positive substances by yeast cells was greater when this organism was pretreated with 56.2 ppm of dodine than when they were

Table 4.—Effect of dodine on the loss of ^{32}P from *Saccharomyces pastorianus* and *Monilinia fructicola*.

Organism	Dodine (ppm)	Initial activity in cells (C/M)	Loss of ^{32}P (C/M) at various intervals (minutes) 5	15	30	60
S. pastorianus	Control	3,117	107	107	112	117
	10.0	3,117	517	746	962	1,321
	31.6	3,117	808	1,422	1,721	1,998
	56.2	3,117	956	1,970	1,998	2,050
M. fructicola	Control	3,689	403	226	145	410
	5.6	3,689	122	213	229	455
	10.0	3,689	268	432	636	989
	17.8	3,689	361	857	1,034	1,280

Table 5.—Comparative effectiveness of various concentrations of dodine and 70% ethanol in removing ninhydrin-positive substances from cells of *Saccharomyces pastorianus* and conidia of *Monilinia fructicola* as indicated by the optical density of the ninhydrin derivatives.

S. pastorianus		*M. fructicola*	
Dodine (ppm)	Optical density	Dodine (ppm)	Optical density
5.62	0.02	3.16	0.05
10.0	0.22	5.62	0.08
31.6	0.62	10.0	0.17
56.2	0.87	17.8	0.47
70% ethanol	0.81	70% ethanol	1.05

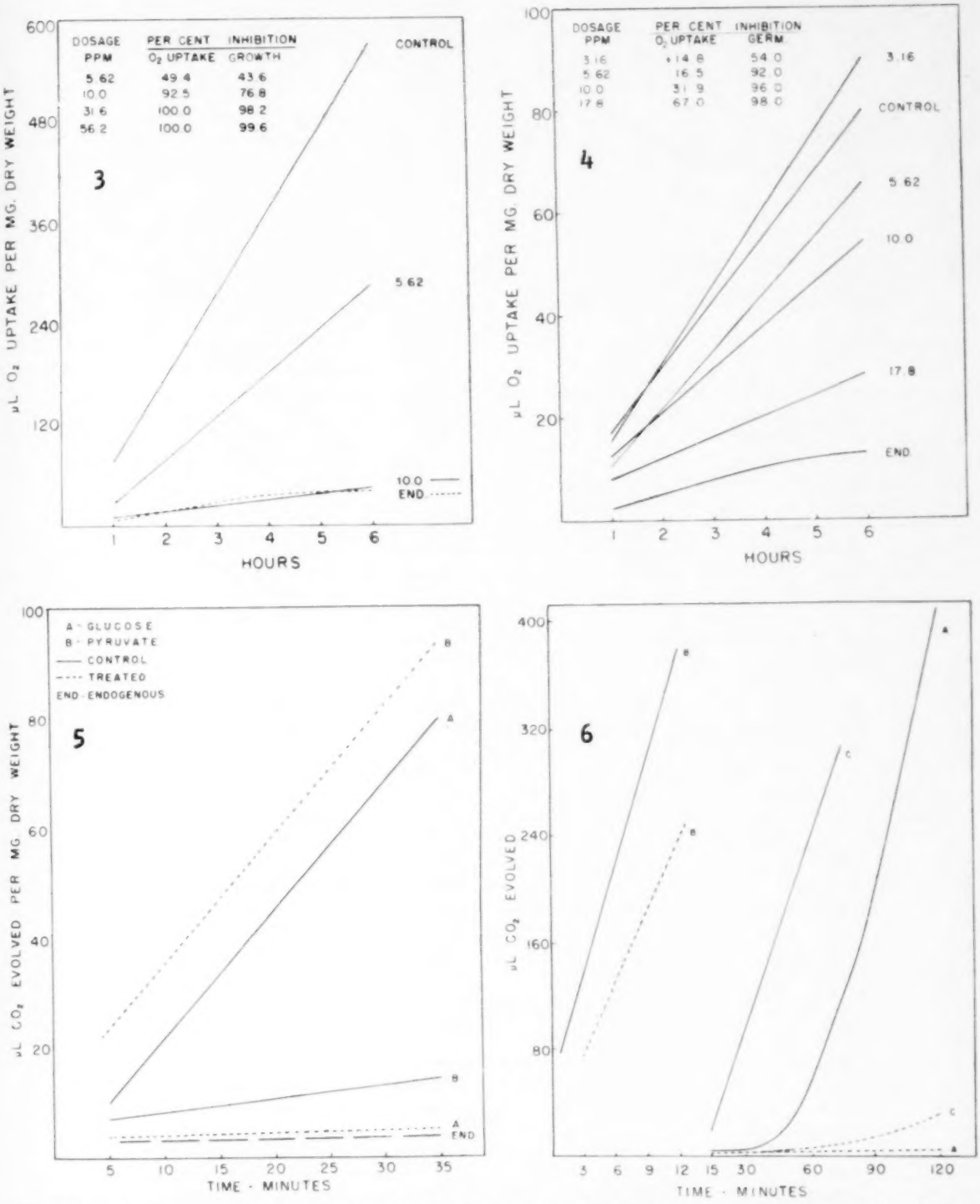


Fig. 3–6.—**3)** The effect of dodine on the oxidation of glucose by cells of *Saccharomyces pastorianus* and its effect on the growth of these cells. **4)** The effect of dodine on the oxidation of glucose by conidia of *Monilinia fructicola* and its effect on the germination of these conidia. **5)** The fermentation of glucose and of pyruvate by untreated cells of *Saccharomyces pastorianus* and by cells pretreated with 31.6 ppm of dodine. **6)** The fermentation of glucose, pyruvate, and fructose-1,6-diphosphate by cell-free extracts prepared from untreated cells of *Saccharomyces pastorianus* and from cells pretreated with 31.6 ppm dodine. Each vessel contained 1 ml of a cell-free extract obtained from yeast cells of 4.9 mg dry weight. A = glucose, B = pyruvate, C = fructose-1,6-diphosphate, solid line = control, dotted line = treated.

Table 6.—Effect of dodine on the uptake of ^{32}P by *Saccharomyces pastorianus* and *Monilinia fructicola*.

Organism	Dodine (ppm)	Initial activity in medium (C/M)	Uptake of ^{32}P (C/M) at various intervals (minutes)			
			5	15	30	60
Saccharomyces pastorianus	Control	2,690	1,215	2,173	2,544	2,583
	10.0	2,690	552	923	1,235	1,857
	31.6	2,690	117	187	188	280
	56.2	2,690	104	104	114	115
			Intervals (minutes)			
			15	30	60	120
Monilinia fructicola	Control	8,152	1,310	2,211	6,066	7,195
	5.6	8,152	1,200	1,482	1,791	1,863
	10.0	8,152	770	1,040	1,572	1,763
	17.8	8,152	1,075	503	388	369

extracted with 70% ethanol. The loss of cellular constituents is closely correlated with toxicity of dodine to the growth of yeast cells.

The phosphorus compounds lost by yeast cells consisted of several types, including inorganic phosphate, sugar phosphates, and compounds with absorption maxima in the vicinity of 260 mμ, probably purine and pyrimidine derivatives. The ninhydrin-positive substances extracted either by 70% ethanol or by dodine consisted almost entirely of 12 commonly occurring amino acids.

Inhibition of spore germination of *M. fructicola* by dodine is not closely correlated with losses of cellular constituents caused by the toxicant. For instance, a 5.62-ppm concentration of the fungicide inhibits germination by 92% but causes hardly any loss of phosphorus compounds and ninhydrin-positive substances (Tables 4, 5). At 17.8 ppm of dodine (ED_{98}), however, there is a considerable loss of these substances.

In both *S. pastorianus* and *M. fructicola*, dodine is more effective in preventing phosphorus uptake than it is in causing the loss of phosphorus compounds taken up prior to exposure to the fungicide (Table 6). In the latter case, the phosphorus taken up and built into cell structure is probably not diffusible and thus would not be lost to the external medium when permeability of the cells is altered by dodine.

Discussion.—It appears that the toxicity of dodine to cells of *S. pastorianus* results from damage to the cellular membrane and also from the inhibition of certain enzymes. Since the cell surface is the first vital site encountered by the toxicant, the primary action probably begins at this locus. Considering the extensive losses of phosphorus compounds and of amino acids from the cells, alteration of permeability alone is probably sufficient to account for the toxicity of dodine to these cells. The remarkable efficacy of dodine in causing yeast to lose cellular constituents is indicated by the fact that a 31.6-ppm concentration of the toxicant will remove nearly as much ninhydrin-positive substance from the cells at 70% ethanol, a solution frequently used in quantitative extraction of free amino acids from cells.

It is presumed that the principal effect of the hydrogen-ion concentration of the external medium in enhancing the toxicity of dodine is that of increasing at the cell surface the number of ionized phosphate and carboxyl groups to which dodine may attach. Since the hydrogen-ion concentration of the medium has a rather marked effect on the toxicity of dodine, it appears that the interaction of the toxicant with these groups at the cell surface plays a critical role in initiating the lethal action of the compound. The alkyl group is also important in determining the biological activity of guanidino compounds. This is indicated by the observation that guanidine acetate homologs of different alkyl chain lengths vary in their toxicity to fungi and higher plants, and by the fact that guanidine acetate itself is not toxic to yeast cells.

The attachment of dodine at the surface of yeast cells probably involves an ionic linkage between the guanidine ion and oppositely charged groups of the cell surface, and also an attachment of the alkyl group to nearby hydrophobic portions. As a result of the attachment of the toxicant to the cell surface it is possible that a mechanical stress is created that causes osmotic damage. A similar explanation of the attachment of surface-active agents to bacterial cells has been given by Dyar (2).

While alteration of permeability of cells of *S. pastorianus* is a very prominent feature in the toxic action of dodine, it has been shown that the fungicide also strongly inhibits critical intracellular enzymes involved in carbohydrate metabolism. These data indicate that the fungicide is capable of penetrating the unaltered membrane of *S. pastorianus* or that it penetrates after it has modified the permeability of the membrane. A number of enzymes in various other phases of metabolism are probably also strongly affected by this toxicant. General protein denaturation, however, is apparently not caused by toxic levels of the fungicide, because the enzyme pyruvate decarboxylase retains much of its activity in treated cells.

It might be argued that such compounds as glucose and HDP are not fermented in extracts from cells treated with dodine, because certain enzymes and coenzymes are lost when these cells are washed following treatment with the toxicant. This seems unlikely, how-

ever, because fermentation of glucose by whole cells treated with dodine and left unwashed, was as strongly inhibited as in the cases where cells were washed following treatment. Furthermore, the lack of cofactors would not appear to be responsible for lack of activity since potassium, magnesium, phosphate, ATP, DPN, acetaldehyde, adenosine, and thiamine pyrophosphate were added to cell-free extracts and also to whole cells in fermentation experiments. In measuring aldolase activity with glucose-6-phosphate as a substrate, the cofactor ATP was added, but there was no significant aldolase activity in whole cells or in cell-free extracts following treatments with dodine. Thus the lack of activity in this case appears to result not from a lack of cofactors but rather from an effect of dodine on enzymes.

The toxic effects of dodine on conidia of *M. fructicola* appear to be somewhat different from those with *S. pastorianus*. Neither loss of cellular constituents nor inhibition of respiration is closely correlated with toxicity of dodine to the conidia. A concentration of 5.62 ppm of dodine, for instance, which strongly inhibits germination (ED_{92}) of conidia of *M. fructicola*, causes practically no loss of phosphorus compounds and ninhydrin-positive substances from the cells. These data indicate that the primary toxicity of cationic surface-active agents does not always involve osmotic damage. A concentration of 17.8 ppm of dodine (ED_{98}), however, does cause considerable loss of cellular constituents. At the higher level of dodine it appears that a toxic mechanism involving alteration of permeability and a mechanism not involving changes in permeability may both be in operation in a population of these conidia.

It is suggested that dodine at the lower concentration inhibits germination of conidia of *M. fructicola* by interfering with vital anionic sites at the cell surface and possibly by interfering with certain vital enzymes other than those involved in the oxidation of glucose. —Department of Botany, University of Maryland, College Park.

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ELIMINATION OF THE INTERNAL CORK VIRUS BY CULTURING APICAL MERISTEMS OF INFECTED SWEETPOTATOES

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SUMMARY

Apical meristems from greenhouse-grown Porto Rico sweetpotato plants infected with internal cork virus were dissected in sodium hypochlorite solution and grown aseptically on a nutrient medium. Young rooted plants that developed in 3–10 months were transplanted to soil. After further growth these were multiplied by vine cuttings for planting in the field. Clonal plants derived from 6 of 7 meristems were free of the internal cork virus as evidenced by no internal necrosis in the harvested roots. Three roots from plants of one meristem clone had symptoms, and these probably developed from one plant reinoculated by a viruliferous insect during the growing season. The plants developed from apical meristems were also free of chlorotic foliage spotting, a syndrome presumed of virus etiology.

Practically all commercially grown sweetpotatoes (*Ipomoea batatas* (L.) Lam.) in the Southeastern States are infected with internal cork virus. It has become exceedingly difficult to locate virus-free "seed stocks." Many choice clones of the Porto Rico variety have become infected and their value thus impaired for producing quality sweetpotatoes. A reliable method of eliminating internal cork virus from infected plants would appreciably facilitate the maintenance and production of virus-free propagative stock.

The demonstration of little or no tobacco mosaic virus on indicator suscepts in apical meristems of systemically invaded tobacco plants (3) prompted other workers to culture meristems from virus-infected plants on artificial media. This procedure has resulted in virus-free plants from infected dahlia (4), potatoes (2, 5), and carnations (7). This suggested its use for developing sweetpotato clones free of the internal cork virus.

Materials and methods.—Apical meristems from infected Porto Rico vines were cultured on a modified Knop medium in March and June, 1958. Infected roots were sprouted in a greenhouse at a minimum of 80°F. Apical meristem tissue was dissected from sprouts 8–10 in. long. The small leaves were cut from the terminal ½ in. of each sprout, and the sprout tip was immersed in Clorox solution (sodium hypochlorite, 5.25 g per L). The meristem was dissected while the forcep-held vine-tip was viewed through a stereoscopic microscope. Usually 2–4 primordial leaves enclosed the meristems. The length of dissected meristems ranged from 0.4 to 1 mm. The meristems were transferred directly to large culture tubes (25×200 mm) containing 25 ml of medium having the following composition per liter: $Ca(NO_3)_2 \cdot 4H_2O$, 0.8 g; KNO_3, 0.2 g; KH_2PO_4, 0.2 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; sucrose, 30.0 g; purified or washed agar, 10.0 g. To each liter of this basal medium was added: 1 ml of ferric citrate (1 g/200 ml), 1 mg pyridoxine, 1 mg thiamine, 5 mg nicotinic acid, and 1 ml of the following solution of salts in 1 liter of water: H_3BO_3, 2.8 g; $MnCl_2 \cdot H_2O$, 1.8 g; $ZnSO_4 \cdot 7H_2O$, 0.2 g; $CuSO_4 \cdot 5H_2O$, 0.08 g; $H_2MO_4 \cdot H_2O$, 0.02 g. Aseptic meristems grew until new plants developed. In a number of cases, living meristems were transferred from desiccated to fresh medium.

Meristems were usually grown in a greenhouse maintained at a minimum of 80°F. The small plants were transferred to water-saturated soil and protected from desiccation with polyethylene bags until they became well established and resumed growth. After young plants were removed from the culture tubes, they were propagated in an area of the greenhouse screened against insects. As the vines lengthened they were multiplied by making vine cuttings.

Results.—The meristems grew into plants rather slowly. Small plants having 2 or 3 leaves developed in 3–10 months. About 10–50 plants from each of 7 meristems (5 cultured in March, and 2 in June, 1958) were planted in the field on May 15, 1959. The sweetpotatoes were interplanted within an acre of land planted half to Irish potatoes and half to tomatoes. An attempt was made to disperse the plants of the meristem clones about the field to reduce the natural spread of internal cork virus in case some clones were infected. When there were more than about 25 plants of a clone, plants of these clones were transplanted at 2 or 3 locations in the field. Few sweetpotatoes are grown in the area selected for the field planting. Infected Porto Rico sprouts were planted on June 15 at the Clayton Experiment Station, about 35 miles distant.

The roots harvested from the meristem clones were compared with roots from infected plants for internal necrosis. The meristem clones were harvested on September 25 and the infected plants on October 23. During this interim roots from the meristem clones were held at 75°F. At harvest, the largest root (about .25 to 1 kg) from clonal plants and infected plants was set aside for the examination following harvest (8). The remaining roots from the meristem clones and a sample from infected plants were incubated at 75°F (6) until January 15, 1960, for the second examination for internal necrosis. Table 1 summarizes the incidence of internal necrosis in roots from meristem clones and infected plants for the 2 dates of examination.

Plants derived from all meristems were initially free of the internal cork virus as indicated by the absence or low incidence of internal root necrosis. Apparently 1 plant of meristem No. 3 became reinfected with the virus during the growing season.

Table 1.—Roots with internal cork symptoms from infected plants and plants derived from apical meristems.

Meristem clone	Roots with internal necrosis	
	10/26/59	1/15/60[a]
1	0/26[b]	0/23[b]
2	0/34	0/53
3	1/28	2/59
4	0/10	0/14
5	0/5	0/20
6	0/5	0/18
7	0/4	0/24
Infected plants	33/59	41/54

[a] Roots of meristem clones stored at 75°F for 112 days and those from infected plants 84 days.

[b] Numerator, roots with symptoms; denominator, roots examined.

Plants of this clone were planted at 2 locations in the field, and the three roots with symptoms (1 observed on the first, and 2 on the second date of examination) came from the same location, whereas roots from the second location (31 roots) were without symptoms. It is postulated that reinfection resulted from inoculation by a windborne viruliferous insect. At the first examination, 56% of the roots from infected plants had symptoms, and 76% had symptoms at the second examination.

Plants of existing Porto Rico clones free of the internal cork virus and those infected with the virus have chlorotic spotting and vein banding foliage symptoms. Foliage of the meristem clones appeared to be free of these chlorotic symptoms, which had already been achieved by Holmes, who propagated stem-tip cuttings (1).

Discussion.—Additional work must be done to determine the length of meristem or vine tip that can be safely used and still obtain plants free of the internal cork virus. Vine tips more than one mm long would probably develop into young plants much more quickly and shorten the interim between meristem dissection and propagation in the field.

The two most serious problems encountered in growing-out the apical meristems were contamination and desiccation of the medium. A large proportion of the meristems were aseptic following the sodium hypochlorite treatment, if the meristem donor was grown in the greenhouse. Fifteen apical meristems were dissected from greenhouse grown sprouts in March, 1958, and 8 of these grew into plants. Vine tips taken from a field plant bed were too heavily contaminated with microorganisms to be disinfested by the sodium hypochlorite solution. In one test, 20 meristem cultures were all contaminated. The same was true of meristems taken from greenhouse grown sprouts heavily infested with aphids. Placing the culture tubes in plastic bags retarded desiccation of the medium, but, because of the high relative humidity, precautions must be taken to prevent the growth of contaminating organisms through the cotton plug or other seal of the culture tube.—Department of Plant Pathology, North Carolina State College, Raleigh.

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CHEMOTHERAPY OF CEREAL RUSTS WITH A NEW ANTIBIOTIC

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SUMMARY

Wheat leaf rust, wheat stem rust, crown rust of oats, and powdery mildew of bean and wheat are inhibited by a new antibiotic (P-9) derived from a *Streptomyces* sp. It is highly potent, persistent, fast acting, and translocatable. Significantly, P-9 is nonphytotoxic.

The use of antibiotics to control cereal rusts has received relatively little attention. Silverman and Hart (3) and Wallen (4) investigated the eradicative properties of a few antibiotics and found that only Acti-dione controlled wheat stem rust in the laboratory. Chemotherapeutic levels were phytotoxic, however. Hacker and Vaughn (1, 2) and Wallen (5) tested various nonphytotoxic derivatives of Acti-dione in both laboratory and field. Although laboratory studies showed that postinoculation treatments were not therapeutic (1), field data indicated that the Acti-dione derivatives protected wheat from stem rust for a considerable time after treatment (1, 2, 5).

A new antibiotic of high chemotherapeutic potency in the control of cereal rusts has been found at the Merck Sharp & Dohme Research Laboratories. The antibiotic, produced by a *Streptomyces* sp., is coded P-9.

Methods and materials.—*Biological assay.*—Unless otherwise stated, the wheat leaf rust syndrome was used as the index of P-9 activity. Little Club wheat

Table 1.—The relative potency of P-9 and Acti-dione in the control of leaf rust on Seneca wheat as measured by the standard eradicant assay. The P-9 used in this experiment is at most 3% pure.

Conc.	Av. no. of pustules per leaf	
ppm.	P-9	Acti-dione
100	0[a]	0[b]
50	1[a]	2[c]
25	6[a]	20[a, d]
10	30[a]	80
5	70[a]	80
1	80[a]	80
0	80	80

[a] Many 0;-type infections.
[b] Severe leaf burn.
[c] Moderate leaf burn.
[d] Slight leaf burn.

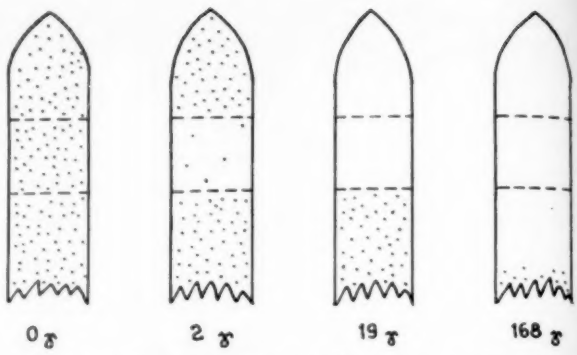

Fig. 1.—The distribution of leaf rust pustules on Seneca wheat leaves treated with P-9 in a localized area. The space between the dotted lines denotes the area of application. The number under each leaf indicates the μg of P-9 solids that were placed on the filter paper attached to the leaf. A 50-ppm solution of the P-9 sample used in this test caused 100% disease reduction in the standard eradicant assay.

(*Triticum compactum* Host) or Seneca wheat (*Triticum aestivum* L.) were the hosts, and *Puccinia rubigo-vera* (D.C.) Wint. f. sp. *tritici* (Erikss.) Carlson, race 9, was the pathogen. Inoculation was accomplished by brushing leaves containing ripe uredinial pustules over the surface of wheat leaves wetted with a solution containing 0.05% Tween 20. The inoculated plants were incubated 18 hours at 70°F and 100% relative humidity. Disease readings were made 10 days after inoculation. The "standard eradicant assay" referred to in the text is based on the treatment of wheat seedlings at the one-leaf stage with an aqueous solution of P-9 in dosage series. The treatments were applied 2 days after inoculation. A De Vilbiss No. 152 atomizer at 15 lb. pressure was used to apply the test samples. The leaves were always sprayed to run-off.

Isolation and partial characterization of P-9.—An isolate of P-9 can be prepared in the following manner: The fermentation beer is concentrated and treated with 7 volumes of methanol, the methanol filtrate is treated with 2 volumes of acetone, and the acetone precipitate can be further purified by cresol extraction. The anti-rust activity is not extracted appreciably into the common organic solvents except phenols. It can be adsorbed on charcoal, clay, florisil, Duolite S-30 resin, carboxylic acid resin, and sulfonic acid resin. Isolates of P-9 are stable at neutral and alkaline pH in aqueous solutions. It is somewhat less stable in acid. It is completely water soluble and dialyzable. Infra-red, nuclear magnetic resonance, and ultraviolet absorption tests on the purest isolate obtained to date were uninformative.

Results.—The experiments reported below were performed with materials in various stages of purification. The purest preparation induced complete eradication at 3 ppm in the standard eradicant assay. Even this material, however, was judged impure by various physical criteria. A comparison of the potency of P-9 with Acti-dione reveals that P-9 is at least 30 times as active (Table 1).

A measure of the persistence and translocation of P-9 is shown by the data in Table 2. First, it will be noted that P-9 protects the foliage for at least 12 days after treatment. Second, the first new leaf (leaf no. 4) formed after treatment is protected from rust infection if the interval between treatment and inoculation is not extended beyond 6 days.

Another expression of P-9 activity above and below the point of application is graphically demonstrated in Fig. 1. P-9 solutions were applied to 1-sq-cm filter pads, which upon drying were clipped to an inoculated leaf 1 cm below the apex. The attached cellulose pads were re-wetted with one drop of a solution containing 0.05% Tween 20 and incubated 3 hours at 70°F in a moist chamber. The treatment was started 2 days after inoculation.

Table 2.—Persistence and translocation of P-9 in Little Club Wheat. P-9 was applied at the dose that produced 90% control in the standard eradicant assay.

Interval, treatment to inoculation (days)	No. leaves fully exposed at time of treatment[a]	No. leaves at time of inoculation	Av. no. of pustules/leaf									
			Treated					Control				
			Leaf position[b]					Leaf position[b]				
			1	2	3	4	5	1	2	3	4	5
6	2	4	0	0	17	3	–	31	60	103	26	–
12	2	5	3	7	275	400	77	52	133	305	432	133

[a] 3rd leaf was just emerging from the whorl of the second leaf at the time of treatment.
[b] Leaf no. 1 is the basal leaf.

Table 3.—The effect of postinoculation wash-off in Little Club wheat. P-9 applied at 4 times the dose that produced 90% control in the standard eradicant assay.

Interval, Inoculation to treatment (days)	Av. no. of pustules per leaf				
	Interval, treatment to wash-off (min)[a]				
	Untreated	15	30	60	Unwashed
1	80	7	2	7	0
2	80	50	30	8	0
3	80	53	53	37	0
4	80	60	57	40	60

[a] A set of untreated inoculated plants were washed at the same intervals noted in the table. In all instances, the number of pustules per leaf was greater than 80.

The effect of pre- and postinoculation wash-off on the antifungal activity of P-9 was investigated to obtain a measure of resistance to weathering. The P-9-treated wheat foliage was washed with distilled water at 15 lb pressure with a De Vilbiss No. 152 atomizer held 3 in. from the leaf surface. The leaves in each pot were washed for 2 minutes. Pre-inoculation wash-off data suggest that P-9 is partially destroyed or diluted in the tissues within a few hours after the spray deposit is removed from the leaf surface. Whereas, the untreated leaves had greater than 80 pustules per leaf, leaves treated with P-9 72 hours prior to inoculation and washed free of the spray deposit just prior to inoculation, had an average of 60 pustules per leaf. On the other hand, the data in Table 3 demonstrate that treatment and wash-off after inoculation gives marked disease control. A leaf surface contact interval as brief as 15 minutes is sufficient for P-9 to inhibit disease development, whether the treatment is 1 or 4 days after inoculation. The same concentration of P-9 was used in both the pre- and postinoculation wash-off experiments.

P-9 also controlled the following diseases: wheat stem rust (*Puccinia graminis* f. sp. *tritici* Erikss. & E. Henn., race 15B) on Seneca wheat, crown rust of oats (*Puccinia coronata* Cda. var. *avenae* Fraser & Led., race 80) on Clinton oats, powdery mildew (*Erysiphe graminis* DC.) on Seneca wheat, and powdery mildew of bean (*Erysiphe polygoni* DC.). In all cases, treatment followed inoculation by 2 days. Oat crown rust was eradicated by the same concentration required to control wheat leaf rust, whereas 2–5 times as much P-9 was needed to eradicate the other diseases. It should be noted that P-9 induced a deformation in bean leaves formed after treatment. Not controlled by P-9 were bean rust (*Uromyces phaseoli* (Pers.) Wint.), early blight of tomato (*Alternaria solani* (Ell. & G. Martin) Sor.), late blight of tomato (*Phytophthora infestans* d By.) and bacterial spot of tomato (*Xanthomonas vesicatoria* (Doidge) Dowson).

Conclusions.—This investigation demonstrates that fermentation broths are capable of yielding translocatable wide-spectrum cereal rusticides of very high potency. Most significantly, P-9 is nonphytotoxic. The mode of action of P-9 is uncertain, but the following points should be noted. P-9 is nonfungitoxic to a number of filamentous fungi and does not inhibit stem rust uredospore germination (personal communication from Dr. John B. Rowell). Furthermore, in the case of stem rust infections, it was observed that, although P-9 markedly reduced the total number of lesions, the few lesions that appeared were of type 4 on a wheat variety (Seneca) that responded to race 15B uredospore infection with type 3 lesions. Also of significance is the fact that P-9 is inhibitory only in situations involving obligate parasitism. These observations suggest that P-9 is not directly fungitoxic to the pathogens in question. Subtle changes in host metabolism induced by P-9 may be responsible for its disease-retarding activity.—Merck Sharp & Dohme Research Laboratories, Rahway, New Jersey.

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A BIOCHEMICAL RESPONSE OF APPLE TISSUES TO FUNGUS INFECTION

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SUMMARY

A fluorescent phenolic compound that increases in concentration in the peel of fruits and leaves of several apple varieties infected with either *Venturia inaequalis* or *Podosphaera leucotricha* is considered to be a product of the host, not the parasite, since it is produced in response to 2 parasitic organisms, occurs in trace quantities in healthy tissues, and is not found in culture filtrates or mycelial extracts of *V. inaequalis*. The increase may be a response to the invasion of these parasites since it was found to occur in mechanically injured tissues.

The external physical symptoms of plant diseases are preceded by and, in part, result from internal biochemical changes. These result from an altered metabolism and can be detected and measured in diseased tissues by changes in the biochemical composition and enzyme activity (3, 6, 11). Herein are presented the results of investigations of such changes in apple tissues infected with *Venturia inaequalis* (Cke.) Wint. and *Podosphaera leucotricha* (E. & E.) Salm.

Materials and methods.—Isolates of *V. inaequalis* were chosen on the basis of pathogenicity on host varieties (12, 14) and growth characteristics in culture. Representative isolates of 3 races were used.

The host plants (Table 1) used were selected for their value in differentiating genes for pathogenicity in *V. inaequalis* (12,14). Host material consisted of young potted trees in the greenhouse and young trees in the orchard. Resistant and susceptible-reacting tissues of the Geneva variety and seedling 384-1 [McIntosh × 45–39 (R12740-7A × Delicious)] were provided by inoculating with appropriate races. Resistant reactions are indicated by reaction class 2 (irregular to regular necrotic or chlorotic lesions without sporulation) and susceptible reactions are indicated by reaction class 4 (extensive lesions with abundant sporulation). A time study was made with inoculated and uninoculated shoots of the Geneva variety. The inoculated shoots provided resistant- and susceptible-reacting tissues; the uninoculated shoots provided healthy tissues. Collections of tissues were made 40, 80, 120, 160, and 200 hours and 12, 18, and 23 days after inoculation.

Tissues were immersed immediately in 50% methanol, at room temperature, on collection and homogenized at room temperature in a Waring Blendor. The filtrate of the homogenate was fractionated with ethyl acetate. The water fraction was further fractionated with acidic lead acetate. Excess lead acetate was removed from the soluble fraction with sodium oxalate. This salt was also used to regenerate the insoluble lead acetate precipitates. Inorganic salts were removed before chromatography by precipitation with ethanol. These three fractions (ethyl acetate soluble; water soluble—lead acetate soluble; water soluble—lead acetate insoluble) were adjusted to 1 ml per 2 g fresh material and chromatographed on Whatman No. 1 filter paper with *n*-butanol–acetic-acid–water (4:1:1) as a descending solvent. Chromatograms were developed with the following sprays (2, 10): brom cresol green, diazotized sulfanilic acid, ferric chloride, HCl-sucrose, Millon's reagent, ninhydrin, and potassium permanganate.

Results.—Barnes (1) had noted an increased concentration of a fluorescent phenolic compound in diseased tissues. In a repeat of his experiments the presence of the compound was confirmed. No other differences were detected between healthy and diseased tissues on chromatograms of the three fractions sprayed with each of the reagents above.

Diseased leaves of the varieties Jonathan, Gallia Beauty, Golden Delicious, Geneva, and 384-1 contained a higher concentration than healthy leaves. In Geneva and 384-1 the concentration was higher in the susceptible host-parasite combination than in the resistant combination. A standard curve of ml of eluate of the fluorescent compound against optical density at a wave length of 310 mμ permitted an estimation of the relative increase in concentration as expressed in units per ml. Ten days after inoculation, the variety 384-1 contained the following concentrations in units per ml: 1.3 in healthy tissues, 18.3 in resistant-reacting tissues, and 25.9 in susceptible-reacting tissues. Thus, there was a 14–19-fold increase in concentration. It seems reasonable to assume that this occurs in infected areas and, therefore, the increases would be much higher since whole leaves were extracted. Observations of chromatograms under ultraviolet light indicated a continued increase in concentration from 40 hours to 23 days after inoculation. In uninoculated tissues, the compound occurred in trace quantities with some increase with age, but concentrations were always lower than in inoculated infected tissues of corresponding age. Since the fruit of each variety is susceptible or resistant in the same respect as the leaves, extracts of the peel of fruits of these varieties were studied and a similar increase in concentration in diseased tissues was noted. Since healthy and diseased fruit were collected in the orchard, no time studies were made. To determine if the increased concentration of this compound is a specific response to infection with *V. inaequalis*, the leaves of Jonathan, Geneva, and 384-1, naturally infected with *Podosphaera leucotricha*, were examined. Increased concentrations were observed in infected tissues, indicating that this is not a specific response to *V. inaequalis*.

To determine if this response was due to a mechani-

Table 1.—Host materials and their reactions to the three races of *Venturia inaequalis*.

Variety or selection	Reaction [a] types induced with races: 1	2	3
Jonathan	4	4	4
Gallia Beauty	4	4	4
Golden Delicious	4	4	4
Geneva	2	2	4
384-1	2	4	2

[a] Reaction class 2 (irregular to regular necrotic or chlorotic lesions without sporulation); reaction class 4 (extensive lesions with abundant sporulation).

cal or biochemical stimulus, leaves of the Geneva variety were rubbed with 600-mesh carborundum to cause visible pigmentation. After 3–5 days they were extracted. No increase in the concentration of the compound was found in 4 replications. Similarly, no increase in concentration could be demonstrated with pin-pricked leaves, pin-pricked fruit, or aphid-injured leaves. Neither could the compound be detected on chromatograms of culture filtrates of mycelial extracts of *V. inaequalis*.

The compound was purified for examination of its chemical and physical properties. Diseased leaves were immersed immediately in 100% ethanol on collection and homogenized in a Waring Blendor at room temperature. The filtrate of this homogenate was passed through a cellulose column. The column was rinsed with 100% ethanol and eluted with water. This fraction was concentrated under reduced pressure, and acidic lead acetate was added. The excess lead acetate was removed from the filtered soluble fraction with sodium oxalate, and the inorganic salts were precipitated with ethanol. The soluble fraction was concentrated again and chromatographed with *n*-butanol–acetic-acid–water (4:1:1). The separated compound was eluted with water and chromatographed and eluted additional times with water and with 50% ethanol for further purification.

The compound is soluble in acetic acid, ethanol, methanol, water, 2,4,6-trimethylpyridine, and aqueous acidic lead acetate; and insoluble in aqueous basic lead acetate, benzene, chloroform, ether, ethyl acetate, toluene, and xylene. Adsorption occurs on charcoal and elution occurs with large quantities of water (recommended by Holoczak). The compound is adsorbed on cellulose from 100% ethanol solutions and is eluted with water. Amberlite IRC-50 charged with acetic acid retains the compound, and dilute acetic acid elutes it. Amberlite IR-4b and acidic and basic Dowex resins were unsatisfactory in that the compound could not be recovered from the effluents and various eluants failed to elute it. It is not known if the compound was not eluted or decomposed by the pH of the resins. The compound exhibits a maximum absorption at 310 mμ and a minimum at 260 mμ (Fig. 1), and a shift of 20–25 mμ of its spectrum to higher wave lengths on the addition of aluminum chloride.

The compound has Rf values of 0.26 in *n*-butanol–acetic-acid–water (4:1:1) and 0.80 in 50% ethanol with no migration in benzene–acetic-acid–water (organic layer of a 6:7:3 mixture) or ammonia-saturated *n*-butanol. The following characteristics were determined after separation by paper chromatography: blue-white with ultraviolet; purple with ferric chloride; orange-brown with diazotized sulfanilic acid; yellow with Millon's reagent; yellow with potassium permanganate; negative with ninhydrin, *p*-anisidine-HCl, and KI-starch. Hydrolysis was partial with boiling for 30 minutes in 0.1*N* NaOH or HCl, and complete in 1*N* solutions. The hydrolysate contained a blue-white fluorescent phenolic compound that is soluble in ether and migrates to an Rf of 0.89 in *n*-butanol–acetic-acid–water (4:1:1) and 0.58 in 50% ethanol.

Discussion.—The compound is phenolic in nature, but is most likely a glycoside or some other derivative since the phenolic product of hydrolysis is soluble in nonpolar solvents, i.e., ether, whereas the parent compound is insoluble. Some flavonoid compounds such as dihydroquercitin and 6-methoxy-benzoxazolinone (9) have spectra similar to that of this compound. Six different glycosides of dihydroquercitin have been isolated from the epidermis of apple fruit (13). The unknown compound exhibits a shift of 20–25 mμ of its spectrum to higher wave lengths on the addition of aluminum chloride. Geissman et al (4) and Harborne (5) report that aluminum also causes a

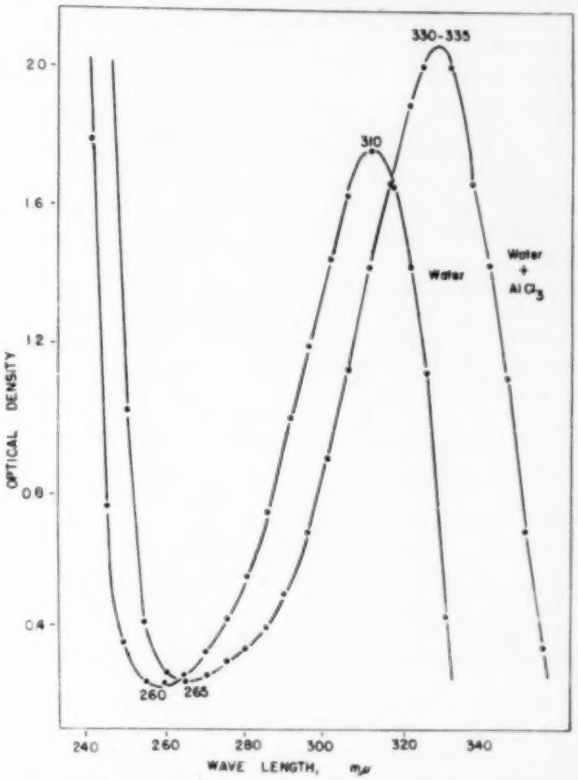

Fig. 1.—The effect of $AlCl_3$ on the absorption spectrum of the fluorescent phenolic compound present in diseased apple tissues. Solvent: water. One drop of 1% aqueous $AlCl_3$ was added to the cuvettes to determine the shift.

shift of 16–35 mμ to higher wave lengths in the spectra of flavonoid compounds with a free 3′4′-dihydroxy grouping, whereas isoflavones with such configurations exhibit no shift.

There would appear to be no correlation of this phenolic compound to those phenolic host metabolites that Kirkham (7, 8) has tested since his materials were extracted with ethyl acetate and this compound is insoluble in that solvent. Kirkham found that the normal phenolic host metabolites (extractable with ethyl acetate and soluble in water) inhibited the development of the pathogen *in vitro* and suppressed the development of sporulation when injected into the host.

The compound is considered to be a product of the host, not of the parasite, since it occurs in trace quantities in healthy tissues, it increases in concentration in tissues infected with either of two parasitic organisms, and it has not been detected on chromatograms of culture filtrates or mycelial extracts of *V. inaequalis*. It may be that the increased concentration is a response to the invasion of certain parasites, but apparently not to mechanical stimuli.

Concentrations were higher in susceptible-reacting tissues, but concentrations per cell may be higher in resistant-reacting tissues, since whole leaves were extracted and resistant lesions are much smaller than susceptible ones. It may be, then, that the quantity of this compound is a significant difference between susceptible- and resistant-reacting tissues. The compound continues to increase in concentration to 23 days after inoculation and would not appear to be an early product of host-parasite interaction. It would seem that the concentration of this compound would be an indirect measurement of a precursor, which would occur in higher concentrations earlier in the process of pathogenesis, and therefore be more significant to that process. Identification of the compound would permit a study of its biosynthesis, metabolism, and toxicity to host and parasite. This would provide for a critical evaluation of the role of this compound and its precursors in biochemical host-parasite interactions.—Department of Botany and Plant Pathology, Purdue University, Lafayette, Indiana.

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CHARACTERISTICS OF PLANT-VIRUS INHIBITORS IN RICE, ORYZA SATIVA

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SUMMARY

Water extracts of rice yield one or more inhibitors that prevent the infection of various susceptible plants by 12 of 15 viruses tested. The data suggest that the inhibitory action is a host response rather than a virus response. A local-lesion bioassay based on the reaction of Pinto bean to infection by tobacco mosaic virus (TMV) was used to characterize the inhibitors. The inhibitors of TMV occurred in fresh, frozen, and dried leaves; flowers; roots; macerated whole grains; refined rice oil; converted rice grains; culms; and rice polish. All 12 rice varieties tested were sources of the inhibitors. The dilution end-point was about 1:3000 (fresh weight) for leaf and root extracts and 1:13,000 (dry weight) for rice polish. Thermal-inactivation studies suggested the presence of 2 or more inhibitors, at least one labile above 60°C and at least 1 stable at 100°. The extracts showed virus inhibition when they were applied to the lower surface of bean leaves and TMV was applied to the upper surface. The extracts prevent infection when applied and then immediately rinsed off 1 or 2 days before application of virus inoculum.

Plant-virus inhibition by plant extracts was first reported by Duggar and Armstrong (4), who demonstrated that the sap of pokeweed, *Phytolacca decandra* L., mixed with tobacco mosaic virus (TMV) prevents infection of tobacco. Other investigators later reported virus inhibition by plant extracts from carnation, *Dianthus caryophyllus* L. (11); spinach, *Spinacea oleracea* L. (9); and New Zealand spinach, *Tetragonia expansa* Thunb. (3). The subject of virus inhibitors from plants has been extensively reviewed (2, 5, 11).

During investigations of the susceptibility of rice, *Oryza sativa* L., to mechanically transmitted cereal viruses (8), the senior author suspected a virus inhibitor in rice. To test this hypothesis, susceptible cereals were inoculated with brome grass mosaic, barley stripe mosaic, or wheat streak mosaic viruses mixed with rice leaf extracts. In addition, Pinto bean leaves were inoculated with TMV mixed with rice extracts. The rice extracts prevented the infection of beans by TMV but failed to prevent infection of wheat or barley by the cereal viruses.

Investigations were therefore initiated to characterize the inhibitor of TMV and to determine the effectiveness of rice extracts in preventing infection by other plant viruses. A preliminary report (1) of some of these investigations and a report (7) on the chemical characteristics of the inhibitor have been published.

Materials and methods.—*Viruses.*—The viruses employed in these investigations were 1) TMV, *Marmor tabaci* H.; 2) tobacco necrosis virus, *Marmor lethale* H.; 3) tobacco ringspot virus, *Annulus tabaci* H.; 4) potato virus X, *Annulus dubius* H.; 5) bean common mosaic virus, *Marmor manifestum* Frandsen; 7) southern bean mosaic virus, *Marmor laesiofacians* Zaumeyer & Harter; 8) vein necrosis virus (strain of alfalfa mosaic virus, *Marmor medicaginis* H.); 9) pod-mottle virus, *Marmor valvolarum* Zaumeyer & Harter; 10) alfalfa yellow mosaic virus, *Marmor medicaginis* var. *flavorians* Zaumeyer; 11) yellow patch virus (strain of alfalfa mosaic virus); 12) wheat streak mosaic virus, *Marmor virgatum* var. *typicum* McKinney; 13) barley stripe mosaic virus; 14) bean red node virus; and 15) a new, unnamed strain of alfalfa mosaic virus.

Tobacco necrosis virus was furnished by R. W. Fulton, TMV by H. H. Thornberry, tobacco ringspot virus by W. B. Allington, potato virus X by R. E. Webb, barley stripe and wheat mosaic viruses by H. H. McKinney, and the others by W. J. Zaumeyer.

Inoculum preparation.—TMV inocula for local-lesion bioassays were prepared from infected Havana 38 tobacco by either of two methods. In one method, the tissue was dried according to the method of McKinney (10). Usually 0.1 g of dried tissue with 5–10 ml of water added was ground in a mortar. Then the extract was expressed through cheese-cloth and diluted to a final volume of 30 ml. In the second method, tissue was frozen and thawed. Then the sap was expressed with a hydraulic press and clarified by heating for 10 minutes at 50°C followed by low-speed centrifugation. The supernatant was diluted with 9 volumes of water and frozen in 10-ml portions.

The inocula for all other viruses were prepared by grinding in a mortar fresh tissue to which 10–20 ml of water had been added. The juice was then squeezed out through 6 layers of cheese-cloth.

Inhibitor preparation.—The inhibitory components were extracted from fresh rice leaf-blade tissue or from rice polish unless otherwise stated. Extracts of leaves were usually prepared by grinding in a mortar 10–20 g of laminae with 5–10 ml of water added, and then squeezing out the juice through 6 layers of cheesecloth. An alternate method consisted of macerating leaf tissue in a blender and squeezing out the juice through cheesecloth. Preparations from rice polish, used in most of the investigations, consisted of 1% suspensions of polish in water. In most experiments, the suspension was allowed to settle for about 2 minutes, and the decantate was used as the inhibitor source. Rice polish was supplied by W. N. Harris, Arkansas Rice Growers Cooperative, Stuttgart, Arkansas.

Measurement of inhibition.—A quantitative test with TMV on beans, based on the local-lesion assay procedure described by Holmes (6), was used for ascertaining the relative inhibition by various rice extracts as compared with appropriate water controls. The two primary leaves of 7- to 10-day-old bean plants (*Phaseolus vulgaris* L. var. UI 111 Pinto) were dusted

with Celite (grade 545) or Carborundum (600 mesh) and then inoculated with TMV by rubbing with the inclined blade of a ground-glass spatula dipped into the inoculum prior to each stroke. During inoculation the leaves were supported by a folded and notched index card backing, 4 layers thick. One leaf of each pair was rubbed with the TMV-inoculum diluted with an equal volume of rice extract, and the other was rubbed with inoculum diluted with an equal volume of water; then, unless stated otherwise, both leaves were rinsed. The inoculated plants were incubated in a greenhouse at about 70–85°F in summer and 70–75° in winter. The numbers of lesions on the inoculated leaves were determined 5–7 days after inoculation. Lesion counting was facilitated by the illumination and slight magnification afforded by a Quebec colony counter. Relative inhibition was determined by the following calculation:

$$\text{Relative inhibition (localized infection)} = -\left\{\frac{\text{mean no. lesions on leaves inoculated with virus-rice extract mixture}}{\text{mean no. lesions on leaves inoculated with virus-water mixture}} \times 100\right\}$$

A semi-quantitative test was used to determine relative inhibition for all other host-virus combinations. The inocula were applied with cheesecloth pads to the Carborundum- or Celite-dusted leaves of the test plants. The plants were then rinsed with tap water. For local-lesion responses, relative inhibition was ascertained by the calculation used in the TMV-Pinto bean bioassay, given previously. For systemic responses, relative inhibition was determined by the following calculation:

$$\text{Relative inhibition} = 100 - \left\{\frac{\text{per cent of plants infected by virus-rice extract mixture}}{\text{per cent of plants infected by virus-water mixture}} \times 100\right\}$$

Results.—*Inhibition of TMV infection of beans by rice extracts.*—Rice extracts contain an inhibitor or inhibitors that prevent infection of beans by TMV. In 40 trials of 10–20 plants each, leaves inoculated with TMV plus rice extract averaged 0–3 lesions whereas opposite primary leaves inoculated with TMV plus an equal volume of water averaged 75–100 lesions.

Inhibition of infection in other host-virus combinations.—Rice extracts were added to inocula of 14 additional plant viruses and relative inhibition calculated for local and systemic infections of several hosts (Table 1). Rice extracts did not inhibit 3 viruses (potato virus X, barley stripe mosaic virus, and wheat streak mosaic virus) on any of the host plants tested. However, rice extracts inhibited infection on at least one host by 11 other viruses. Rice extracts inhibited infection by 10 viruses known to infect beans, regardless of whether the infection was systemic or local. Of these 10 viruses, 7 are also known to infect cowpeas. On cowpeas, the relative inhibition of these 7 viruses, as shown in Table 1, was 94, 66, 64, 58, 56, 13, and 9, indicating that one virus was inhibited, 4 others were partially inhibited, and 2 others were inhibited but slightly if at all.

Location of inhibitor in rice.—Extracts of various portions of rice (var. Caloro and Zenith) were found to be sources of TMV inhibitor when tested by means of the bioassay. The relative inhibition was 98–100 with water extracts of fresh, frozen, sun-dried, or oven-dried (104°F) leaves from 5- and 14-week-old plants; roots; flowers; macerated whole grains; and rice polish. The relative inhibition was 83 with water extracts of culm tissue; 63 with converted rice (Minute Rice brand); and 57 with refined rice oil, which was slightly phytotoxic to bean. Crude rice oil preparations were so phytotoxic that the relative inhibition could not be ascertained.

Rice varieties.—All 12 of the rice varieties or plant introductions tested were sources of TMV inhibitor. The varieties or introductions were: P. I. 184675, P. I. 184676, P. I. 184676A, India 1, La Crosse, Wag-wag, Sha-tiao-tsoa, Rexoro, Paddy NP-124, Paddy T-3, Zenith, and Caloro. The average relative inhibition for all varieties was 97% as determined by the TMV-Bean bioassay.

Post-inoculation rinsing.—Rice extracts were inhibitory to TMV infection of beans, whether or not the virus-extract mixture was rinsed off leaves immediately after inoculation. For example, in the varietal test mentioned previously, in which relative inhibition averaged 97% for rinsed leaves, it averaged 98% for unrinsed leaves.

Dilution end-point.—The dilution end-point of active inhibitor was more than 1:3000 for leaf and root extracts (Fig. 1) and 1:13,000 for rice polish extracts (Fig. 2). Dilutions of root and leaf extracts were based on fresh weight, and those of rice polish on dry weight. The dilutions at which loss in activity was 50% were 1:350 for root and leaf extracts and 1:4,500 for rice polish extracts.

Thermal inactivation.—Data concerning thermal inactivation suggest that there may be 2 or more inhibitory components, of which at least 1 is stable at 100°C and 1 is heat labile. For example, relative inhibition of TMV infection on beans in 5 trials with unheated leaf and polish extracts averaged 100, whereas the relative inhibition in these same 5 trials was 81, 3, 85, 58, and 91 for leaf extracts and 52, 58, 45, 22, and 85 for polish extracts maintained at 100° for 10 minutes. At 60, 70, 80, and 90° the relative inhibition varied between 40 and 70, showing a loss in some inhibitor activity, but not all. However, none of the 10-minute exposures between 60 and 100° and not even a 30-minute exposure at 100° destroyed all inhibitory activity.

Table 1.—Effect of rice polish extracts in the inocula of 15 plant viruses on the infection of various susceptible plants.

Virus and host	Type of infection	Infection with virus plus: water [a]	Infection with virus plus: rice extract [a]	Av. relative inhibition [b]
Tobacco ringspot				
Bean (Pinto) [c]	local	75	2	97
N. tabacum (Samsun)	systemic	3/3	3/3	0
N. tabacum × *N. glutinosa*	systemic	3/3	3/3	0
Cowpea (black) =	local	63	65	0
Cowpea (black-eye)	local	45	41	9
Alfalfa yellow mosaic				
Bean (Pinto)	local	60	1	99
Cowpea (black)	local	79	35	56
Yellow patch strain of alfalfa mosaic				
Bean (Refugee)	local	34	22	35
Cowpea (black)	local	12	5	58
Alfalfa mosaic (new unnamed strain)				
Bean (Pinto)	local	24	2	92
Bean (Pinto)	systemic	20/23	1/24	95
Cowpea (black)	local	11	4	64
Cowpea (black)	systemic	22/27	21/28	14
Barley stripe mosaic				
Barley (Atzel)	systemic	30/30	30/30	0
Wheat streak mosaic				
Wheat (Pawnee)	systemic	30/30	30/30	0
Vein necrosis strain of alfalfa mosaic				
Bean (Pinto)	local	12	0	100
Cowpea (black)	local	3	1	66
Common bean mosaic				
Bean (Pinto)	systemic	19/26	0/26	100
Bean yellow mosaic				
Bean (Pinto)	systemic	24/24	0/22	100
Bean (top crop)	systemic	19/19	17/17	0
Soybean (Hawkeye)	systemic	4/5	2/5	50
Southern bean mosaic				
Bean (Pinto)	local	243	12	95
Bean pod mottle				
Bean (Pinto)	local	99	12	88
Soybean (Hawkeye)	systemic	10/10	10/10	0
Bean red node				
Bean (Pinto)	local	103	1	99
Bean (Pinto)	systemic	10/10	0/10	100
Cowpea (black)	local	16	1	94
Tobacco necrosis				
Bean (Pinto)	local	10	0	100
	local	19	0	100
	local	22	0	100
Cowpea (black)	local	16	14	13
Cowpea (black-eye)	local	45	41	9
Potato X				
N. tabacum (Samsun)	systemic	5/5	5/5	0
Gomphrena globosa	local	43	40	7
Physalis floridana	systemic	5/5	5/5	0
Chenopodium amaranticolor	local	35	37	0
Capsicum annuum (Cayenne)	systemic	5/5	5/5	0
Tobacco mosaic				
Capsicum annuum (Cayenne)	systemic	14/14	20/20	0
C. frutescens (California Wonder)	local	10	10	0
Petunia hybrida (Snowball)	systemic	5/5	7/7	0
Datura stramonium	local	100 [d]	100 [d]	0
Chenopodium amaranticolor	local	25 [d]	25 [d]	0
Tetragonia expansa	systemic	7/7	8/8	0
Lycopersicon esculentum (Marglobe)	systemic	5/5	5/5	0
Gomphrena globosa	local	30	32	0
N. tabacum (Xanthi)	local	72	69	5
N. tabacum (Xanthi)	local	271	61	78

[a] Whole numbers = average number of local lesions in 1–3 trials of 10–20 plants each. Fractions = total number of plants infected per total number inoculated in 1–3 trials.

[b] Formulae for calculation of Relative Inhibition described under "Methods and materials."

[c] The strain of Pinto used was U. of Idaho 111.

[d] Estimate.

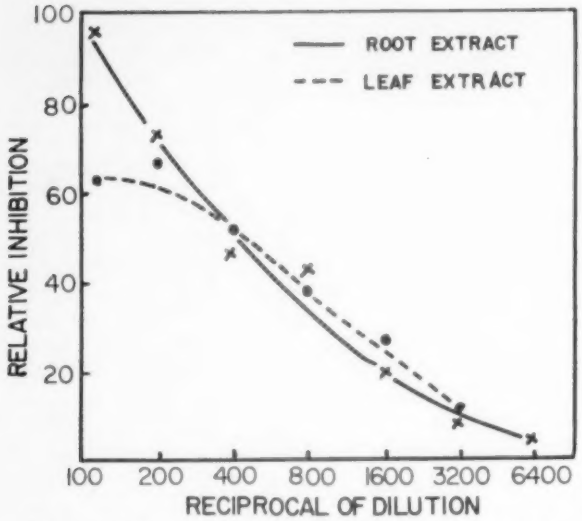

Fig. 1.—Dilution end-point of tobacco mosaic virus inhibitor in rice leaf and root extracts (fresh weight).

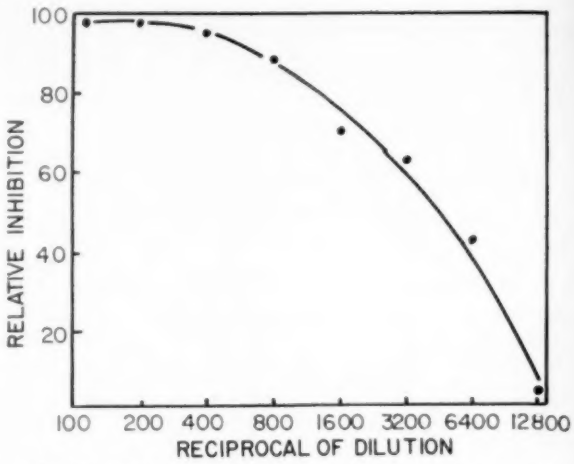

Fig. 2.—Dilution end-point of tobacco mosaic virus inhibitor in rice polish (dry weight).

Translocation.—Extracts of rice leaves or polish were applied to soil, to the first internode area of beans with a hypodermic needle and syringe, or to the lower surface of bean leaves, and the TMV was applied to the upper surface of primary leaves. In 2 trials, rice extracts were not inhibitory when applied to the soil 1, 2, or 3 days before inoculation, indicating the inhibitor is not translocated to the primary leaves. Similarly, extracts were not inhibitory, in two trials of 15 plants each, when injected into the first internode area 1 or 20 hours prior to inoculation. However, the relative inhibition in 3 trials was 62, 77, and 86 when the extracts were applied to the lower surface and immediately rinsed off ½ to 1 hour prior to the inoculation of the upper surface.

Application of inhibitors before and after inoculation.—Rice extracts applied before or after the application of virus inoculum inhibited infection of beans by TMV. Pre-inoculation sprays or dips in rice extracts were more inhibitory than post-inoculation applications. In 3 trials, when rice-polish extracts were applied and rinsed off immediately at 148, 72, 48, 24, and 6 hours before inoculation, relative inhibition was respectively 20, 57, 77, 88, 98, and 97 as compared with 100 when the virus and extract were applied simultaneously. In 2 trials the relative inhibition for extracts applied 2, 6, 24, and 48 hours after inoculation was respectively 44, 37, 49, and 14. These data, obtained from comparisons between groups of rice-extract-treated and untreated plants, suggest that rice extracts may prevent TMV infection of beans when applied up to 3 days before inoculation or may inhibit or retard virus multiplication when applied up to 1 day after inoculation.

Discussion.—Several lines of evidence indicate that the inhibitor or inhibitors in rice act on the host plant rather than on the virus. First, if the action were on the virus, one would expect that mixing a virus inoculum with the inhibitor would inhibit infection of all susceptible hosts. The data (Table 1) show to the contrary, that inhibition occurs on beans, in some cases on cowpeas, and in one instance on *Nicotiana tabacum* var. Xanthi, but not on any of the other hosts. Second, the inhibition occurred whether the inhibitor was applied to the upper or lower leaf surface, although the virus was applied only to the upper surface. Third, infection was inhibited by extracts applied and immediately rinsed off when the virus was applied 1 or 2 days later. One must assume, however, that the rinsing actually removes inhibitor from the leaf surface because any inhibitor remaining on the leaf could be mixed with the virus at the time of virus application. However, no such mixing on the leaf surface could occur when the inhibitor is placed on the lower surface and the virus on the upper.

Another point of interest is the suggestion of varietal specificity, since bean yellow mosaic virus was inhibited on the Pinto bean but not on the Topcrop variety.—Plant Quarantine Division, U. S. Plant Introduction Station, Glenn Dale, Maryland, and Crops Research Division, Plant Industry Station, Beltsville, Maryland.

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THE INFLUENCE OF CATION COMPETITION, TIME, AND TEMPERATURE ON THE UPTAKE OF STREPTOMYCIN BY FOLIAGE

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SUMMARY

Leaves of apple, *Coleus*, and other plants, homogenized in aqueous solutions containing streptomycin and metallic cations KCl, NaCl, $MnCl_2$, $CaCl_2$, $AlCl_3$, and others, clearly demonstrated a competition between the metallic cations and the organic cation streptomycin for adsorption sites in homogenized leaf tissue. Excised apple, bean, and *Coleus* leaves absorbed streptomycin in significant quantities from a water agar containing the drug. The quantity absorbed was influenced by length of exposure (time), specific surface exposed to the agar (upper or lower), and temperature. Generally, the longer the exposure period, the more streptomycin was absorbed. Bean and *Coleus* foliage both absorbed more streptomycin through the lower surface, whereas apple leaves absorbed more through the upper surface, which is devoid of stomatal openings. Temperature coefficients ($Q_{10's}$) of 2.0 and 2.4 for bean and apple leaves, respectively, were obtained for streptomycin absorption by lower leaf surfaces over a relatively wide temperature range of 18°C. These Q_{10} values suggest that foliar absorption may be metabolically controlled, at least in part.

Introduction.—The initial experiments described in this paper were designed to ascertain whether ion competition between streptomycin and inorganic cations was peculiar to the K^+ and Mn^{++} ions or if this was a general phenomenon exhibited by inorganic cations. The remainder of this report is devoted to exploring the influence of such factors as time (duration of exposure), temperature, and surface specificity (upper or lower surface) on the absorption of streptomycin by foliage.

Review of literature.—Shortly after initial laboratory and field experiments had demonstrated the efficacy of certain antibiotics against a number of bacterial phytopathogens, it was also shown that these large organic molecules were capable of being translocated in the vascular system of the plant. The uptake and subsequent translocation from the point of entry of streptomycin, for example, have been demonstrated in a diverse group of monocotyledonous and dicotyledonous plants (7). Studies of antibiotic absorption at the cellular level have been conducted by Pramer (16, 17) and Litwack and Pramer (14), using the large-celled fresh-water alga *Nitella clavata*. Their results suggested that the absorption of streptomycin was, at least in part, an active process mediated by respiratory energy and that accumulation of this compound was possible against a concentration gradient. Accumulation of streptomycin in foliage against a gradient, via root-system absorption, has been reported for peach seedlings (5) and for tomato and lettuce plants (3).

Indirect evidence of improved absorption of streptomycin by apple foliage with adjuvants, e.g., humectants and cuticle solvents (6), suggested a new approach to heightening the systemicity of organic spray compounds. Direct evidence of the activity of humectants in increasing absorption of streptomycin was first presented by Gray in experiments using glycerin (12). Improved absorption of this antibiotic in bean foliage was obtained with certain growth regulators (9). Subsequent studies showed that streptomycin could penetrate foliar surfaces devoid of stomatal openings (4, 7).

A number of workers, but primarily Ark and his group (1, 2), demonstrated that the absorption of the cationic molecule streptomycin by plant tissues is influenced by the ion exchange phenomenon. It is apparent from these and other studies that certain antibiotics are strongly adsorbed by colloidal systems, both protoplasm (11, 18) and soil (10). The ions found to be most effective in competing with streptomycin for adsorbing sites were K^+ and Mn^{++}.

Materials and methods.—The evaluation of competition between metallic cations and streptomycin for adsorbing sites on plant tissue employed 2.5-g samples of apple, peach, carnation, *Coleus*, pepper, and bean foliage. The samples were homogenized in a Virtis apparatus for 5 minutes in solutions containing 1% of the ion to be tested (in the chloride form) and streptomycin hydrochloride at 4 μg/ml. The homogenate was centrifuged and the supernatant assayed for streptomycin activity with the stainless-steel cylinder-agar-diffusion method described in detail by the author (7, 8). Sensitivity of the assay is 0.3 μg/ml streptomycin.

Yarwood (21) indicated that excised leaves could be maintained in nutrient solutions for extended periods. With Petri dishes as humid absorption chambers, apple leaves were floated in water containing 25 μg/ml of streptomycin. It was immediately apparent, however, that the treating solution frequently washed over the upper surface of the floating leaf. To preclude

Table 1.—Effect of cations on streptomycin adsorption by apple leaf homogenate.

Cationic source [a]	pH	Activity [b] (ppm) of supernatant liquid
H_2O	6.7	0.35
$CaCl_2$	6.0	0.74
KCl	6.7	1.20
NaCl	6.7	1.32
$MnCl_2$	5.5	3.75

[a] Salt solution, 1%.
[b] Streptomycin activity, 4 ppm.

absorption by any surface other than the one in contact with the liquid, a 0.5% water agar was used. Similarly, the agar contained streptomycin at 25 μg/ml upon which were placed 1 or 2 leaves per plate while the agar was still fluid. Petri plates were filled to about half their capacity.

Apple, bean, pepper, and *Coleus* leaves, upper and lower surfaces, were placed, alternately, in contact with the agar and permitted to absorb streptomycin at 10, 28, and 37°C for 24 and 48 hours.

The amount of antibiotic absorbed by the intact leaves was determined by the cylinder-agar-diffusion method (7, 8). All leaves were washed for 1 hour in running water before preparation for assay. The sample size was 1 g of fresh leaf tissue taken from a composited 10–12-leaf sample. The leaf tissue was frozen and the cellular fluids were expressed in a Carver press. Temperatures were maintained at ±0.5°C in a low-temperature incubator.

It was assumed that the excised leaves were transpiring at least in those instances where stomatal openings were adjacent to the atmosphere of the Petri dish. Further, it was conceivable that this transpiration could influence uptake of streptomycin by the surface in contact with the agar. To test these assumptions, Petri plates were filled to within 1/8 in. of their rims, thereby reducing the volume of air that had to be brought to water-vapor equilibrium with the leaf tissues. The lids of the Petri plates were inverted and a seal effected at the rim of the dish with petroleum jelly. This minimized the loss of water vapor from the dish.

Results.—Table 1 indicates that all of the metallic salts used could compete with the organic cation streptomycin for positions on the adsorbing surfaces of homogenized leaf tissue. Similar experiments were conducted with pepper, peach, carnation, *Coleus*, and bean leaf tissue, and these provided essentially the same results. The different plant species varied from one another in the amount of streptomycin adsorbed and the amount that could be exchanged from the surface by the metallic cations. In addition to the cations noted in Table 1, $LaCl_3$, $MgCl_2$, $FeCl_3$, $BaCl_2$, and $CuCl_2$ were also examined, and these cations too competed with streptomycin for sites on the various leaf tissues. From these results it appears that the competitive effect observed initially with K^+ and Mn^{++} was a general phenomenon that would influence the adsorption, and perhaps the absorption and subsequent translocation, of many cationic or imphoteric organic compounds.

Table 2.—Effect of temperature and time on streptomycin[a] absorption by upper and lower apple leaf surfaces.

Leaf surface	Temp. (°C)	Time (hr)	Absorption (ppm)
Upper	10°	24	4.9
Lower	10°	24	1.9[b]
Upper	10°	48	10.5
Lower	10°	48	6.7
Upper	28°	24	14.4
Lower	28°	24	8.6[b]
Upper	28°	48	19.6
Lower	28°	48	19.9

[a] Streptomycin concentration in agar was 25 ppm.
[b] Q_{10} for rise in temperature of 18°C was 2.40.

In Table 2 the effects of time, temperature, and specific surface in contact with the streptomycin-impregnated agar are all apparent as significant factors, influencing the absorption of streptomycin by apple foliage. From these data there is little doubt that: a) absorption proceeds through the upper leaf surface despite the fact that there are no stomatal openings present, b) absorption increases with the 18°C increase in temperature, c) absorption is greater through the upper than through the lower surface, d) the temperature-dependent increase seems to be more significant when absorption is proceeding through the lower surface, e) the Q_{10} obtained for a 24-hour absorption by lower leaf surfaces is indicative of a reaction(s) that is, at least in part, biochemical in nature, and f) the longer the leaf is exposed to streptomycin, the greater the quantity absorbed. It might also be pointed out that the difference in absorption between the upper and lower surfaces is not nearly as appreciable after 48 hours as that noted following the initial 24-hour exposure. This is particularly true at 28° where, in fact, there is no difference.

From the data in Table 3 it seems a) that streptomycin absorption by bean foliage increases with increasing temperatures, b) and Q_{10} obtained for a 24-hour absorption is indicative of a reaction(s) that is, at least in part, biochemical in nature, c) streptomycin absorption is greater via the lower surface than the upper (a reversal of the situation noted with apple foliage), and d) the variation in absorption between upper and lower surfaces is not apparent at the lowest temperature of the experiment, 10°C.

Table 3.—Effect of temperature on streptomycin[a] absorption by excised bean foliage.

Leaf surface	Temp. (°C)	Absorption[b] (ppm)
Upper	10°	2.8
Lower	10°	2.5[c]
Upper	28°	5.3
Lower	28°	9.1[c]
Upper	37°	8.3
Lower	37°	12.1

[a] Streptomycin concentration in agar was 25 ppm.
[b] Absorption for 24 hours.
[c] Q_{10} for the rise in temperature of 18°C was 2.0.

Table 4.—Streptomycin[a] absorption by excised *Coleus* leaves.

Leaf surface	Leaf color	Absorption[b] (ppm)
Upper	Green	3.8
Lower	Green	4.1
Upper	Red	5.8
Lower	Red	8.2

[a] Streptomycin concentration in agar was 25 ppm.
[b] Absorption for 48 hours, at 28°C.

The *Coleus* leaves treated in the experiment recorded in Table 4 were of similar size, age, and position, and in these respects the data should be comparable. It is apparent that the red-leaved species absorbed more streptomycin than the green-leaved one. Furthermore, *Coleus*, which, like apple, has stomata only on the lower surface, is unlike the latter in that it absorbed more streptomycin via the lower surface.

The data in Table 5 describe the influence that relative humidity of the atmosphere in the Petri dishes had on the absorption of streptomycin. It seems that the differences between the two atmospheres (conventional and sealed) were either quite small or not significant enough to vary streptomycin absorption.

It should be pointed out that bean leaves have stomata on both surfaces, though there are 7 times as many on the lower surface as on the upper. Now, if stomatal transpiration, which accounts for 90% of foliar transpiration (15), had been a significant factor in streptomycin absorption, uptake should have been greater in Petri dishes where the lids were placed in the conventio al manner. Under these conditions there was an appreciable loss of water vapor from the plate, whereas water loss from the sealed plates (inverted lids) was negligible. Furthermore, if stomatal transpiration was influencing absorption one would expect the greatest quantities of streptomycin to be in those leaves whose upper surfaces were in contact with the agar. Here, 7 times as many stomata would have been functioning (28,000/cm^2 as opposed to 4,000/cm^2) (15), yet there was less streptomycin absorbed where the upper surface was in contact with the agar.

Discussion.—The foregoing experiments have shed some additional light on the nature of the foliar uptake of relatively large cationic organic molecules and have tended to substantiate some recent findings regarding foliar absorption of the comparatively small inorganic cations. The researches of Zukel et al (22) concerning the foliar absorption of maleic hydrazide (MH) indicated that relative humidity was the greatest single factor affecting absorption rate. They also indicated that temperature at controlled high humidities had less of an effect. In a more recent effort, however, Smith et al (19) showed that tomato plants absorbed MH about 60% as fast at 10°C as at 32°C. In the current experiments, relative humidity was not a limiting factor, and thus the influence of temperature on absorption was more apparent.

It is quite possible that relative humidity and temperature influence the absorption process of foliage in distinctly different ways. It has been suggested (7) that the improvement in foliar absorption resulting from high relative humidity or from a humectant-type compound such as glycerin is due to hydration of the cuticle—specifically, hydration of cellulose and cutin, the hydrophyllic components of the cuticle, and perhaps the pectic layer that separates it from the epidermal cells. Hydration would result in a swelling of the cuticle, and its micellar structure would be forced apart, lending a porosity to the cuticular barrier. Under such a scheme it is relatively simple to visualize the movement of water-soluble organic compounds into leaf tissue.

The effect of temperature on absorption, in this study, might be explained if at least a part of the absorption is visualized as an active one. Direct evidence of active absorption of streptomycin by *Nitella* (14, 16, 17) supports this contention. Further, the indirect evidence of improved streptomycin absorption in response to low concentrations of growth regulators, naphthyl acetamide and gibberellic acid, is also suggestive of a metabolically controlled phenomenon (9).

In the present work, temperature coefficients (Q_{10}'s) of 2.40 and 2.00 were obtained for the absorption of streptomycin over a relatively wide temperature range (Tables 2, 3) by apple and bean foliage, respectively. Values such as these are more indicative of biochemically controlled reactions than a purely physical one such as diffusion.

It should also be noted that the relatively high Q_{10}'s were obtained when absorption was proceeding through the lower surface. A preponderance of stomatal openings at this surface, in both instances, strongly suggests these openings as a significant factor in absorption. This factor may be more apparent than real, however. It is conceivable that thickness of cuticle, trichomes, protuberant veins (the latter 2 factors providing increased specific surface) as well as the presence of specialized parenchymatous tissue composed of thin-walled cells influences absorption more profoundly.

Direct penetration of the cuticle of the upper surfaces of apple leaves, devoid of stomatal openings, precludes these foliar orifices as requisite entryways. In fact, the greater absorption of streptomycin by the upper surfaces of apple leaves establishes cuticular penetration as a significant route for the movement of quantities of organic substances into leaf tissues. Similar data have recently been presented for P^{32} (20) and

Table 5.—Influence of relative humidity on absorption of streptomycin[a] by excised bean leaves.

Leaf surface	Petri dish (rel. humidity)	Absorption[b] (ppm)
Upper	conventional	5.7
Lower	conventional	9.5
Upper	sealed	6.3
Lower	sealed	9.5

[a] Streptomycin concentration in agar was 25 ppm.
[b] Absorption for 24 hours, at 28°C.

Co^{60} (13), and together with those presented herein it appears that comparatively large organic molecules as well as the smaller cations and anions are capable of analogous movement into foliage, i.e., through the cuticle.

The exchange phenomenon exhibited between streptomycin and a number of metallic cations for adsorption sites in homogenized plant tissue has a number of implications. First, it can be assumed that this phenomenon is a general one, not restricted to a few cations. The presence of metallic cations in other spray materials (pesticide mixtures) as well as residues in water and in the spray equipment may be expected to interfere with the adsorption of organic molecules that are either cationic or amphoteric. From the data presented in Table 4, it is apparent that varieties within a plant species differ in the extent to which they will absorb a cationic organic compound. Perhaps the quantity and availability of negative charges at the surface account for this difference. The predominating charge and the arrangement of the adsorbing colloidal system may also account for the tenacity with which the compound is held, once inside the plant. Finally, the kind and quantity of endogenous cations present in the plant tissue may also influence adsorption and subsequent movement of the adsorbed compound.—Department of Horticulture, University of Missouri.

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TESTS WITH VOLATILE FUNGICIDES IN PACKAGES OF CITRUS FRUITS DURING SHIPMENT TO EASTERN MARKETS

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SUMMARY

Where results were statistically separable, commercially handled citrus fruit treated with biphenyl had less decay, soilage, and chemical injury than those treated with ammonia. Both treatments were significantly superior to no treatment in control of decay. In these tests, biphenyl caused no apparent injury of the fruit; ammonia, however, under conditions that produced excessive condensation of moisture, occasionally injured the rind of lemons and navel oranges. This injury was not seen on Valencia oranges.

With artificially inoculated fruit, treatments varied in their relative efficacy in control of decay. For lemons, biphenyl was superior to ammonia. For navel oranges, the results with ammonia and biphenyl could not be separated statistically. For Valencia oranges, ammonia was distinctly better than biphenyl. For all varieties, both treatments were significantly superior to no treatment.

Recent changes in methods of packaging citrus fruit have necessitated changes in decay control measures during transit and storage. Fruits are no longer individually wrapped in tissue paper and packed in wooden boxes, but are placed unwrapped in fiberboard cartons and protected from blue-green mold decay by the volatile emissions from 2 sheets of kraft paper impregnated with biphenyl (7).

Continuous screening for a more effective chemical fungicide than biphenyl has been in progress in this department for a number of years. Of the many chemicals screened, ammonia continued to show the most promise in controlling decay with minimal injury (8, 9, 10), and attempts were made to adapt it for use in cartons (4, 5). With no stipulation as to residue, ammonia was exempted from a requirement for a tolerance on citrus (3). Tests with biphenyl and ammonia were therefore made to compare the effectiveness of the two chemicals for control of blue-green mold decay caused by *Penicillium italicum* Wehmer and *P. digitatum* Sacc. during commercial shipments. This paper reports the results and some of the problems involved.

Experimental procedure.—Cartons of experimental fruit were hand-packed in the packing house, and the protective chemicals were introduced. Labeled cartons were placed in refrigerated railroad cars or trucks and sent to receiving personnel in Kansas City, Chicago, or New York. Reports on fruit decay, soilage, black buttons, and injuries were mailed back to the Citrus Experiment Station for statistical analysis.

Over 150 experimental shipments were made by rail and truck from various packing houses to the cities mentioned; most were by rail to New York City. The fruit was randomized into the required number of containers and, if to be inoculated with blue-green mold spores, was first scratched to produce a wound 1 mm deep and 1 cm long, and showered with dry spores of *Penicillium italicum* and *P. digitatum*. The fungicidal treatment materials were: 1) two standard biphenyl kraft sheets measuring 11 × 17 in. and each containing 2.35 g of biphenyl; 2) double paper sheets, also 11 × 17 in., one impregnated with an ammonium salt, the other with an alkali to liberate gaseous ammonia from the other sheet when in the moist atmosphere of the carton of fruit; and 3) dry compressed tablets of the ammonia-evolving chemicals placed in porous paper packets of 9 tablets each (4, 5, 8). The ammonia-emitting materials were placed horizontally between layers of fruit, and the biphenyl sheets were placed vertically on the inside surfaces of the carton. Each lot of treated fruit, in 3–6-carton replications, was placed in a vertical stack in the refrigerator car or truck.

Arrangements were made for receiving and inspecting the fruit shipments. Report forms used at shipping, receiving, and final inspection included such items as picking dates, packing-house treatments, icing conditions, carton type and brand, packing, precooling, and shipping temperatures, and decay control treatments.

At the terminal markets, initial inspection for decay and injury was made for some experimental shipments, and all fruits were then stored for one week. They were then thoroughly examined for decay, black button injury, and soilage.

Decay was grouped into two categories: that caused by *Penicillium* spp., and that caused by other molds. Since over 95% of all decay found was caused by the *Penicillium* spp., only such were included for analysis. Chemical surface injury, if present, was recorded as slight, moderate, or severe. Another index of injury was the color of the buttons (6). The number of fruits whose normally green buttons were discolored, blackened, or dropped off was recorded. Soilage is defined as the dirtying of adjacent sound fruit by spores from an infected fruit (2). A fruit was considered soiled if 1 cm or more in diameter of its surface area was colored with blue or green spores.

This paper reports the results of 9 experiments, in-

Table 1.—Statistical analysis (in angles) of 6 experiments with uninjured uninoculated fruit.

Source of variation [a]	Expt. 1 12 cars navels	Expt. 2 11 trucks navels	Expt. 3 8 cars lemons	Expt. 4 8 cars lemons	Expt. 5 11 trucks lemons	Expt. 6 12 cars Valencias
			Decay			
H		NS	NS	NS	NS	***
S(H)	***	***	**	***	***	***
T	***	***	***	***	***	*
T × H		NS	NS	NS	NS	NS
T × S(H)	NS	NS	(E)	NS	NS	*
			Soilage			
H		NS	NS	NS	NS	*
S(H)	***	***	*	*	***	***
T	***	***	***	*	*	
T × H		NS	NS	NS	NS	NS
T × S(H)	(E)	NS	(E)	(E)	NS	(E)
			Black Button			
H		NS	NS	NS	NS	*
S(H)	***	***	**	**	**	NS
T	*	NS	*	**	NS	***
T × H		***	NS	NS	**	NS
T × S(H)	(E)	NS	(E)	(E)	**	(E)

[a] *Code symbols:* H, houses; S(H), shipments or shipments within house if more than 1 house is concerned; T, treatments; T × H, treatment by house interaction; T × S(H), treatment by shipment interaction or treatment by shipment within house interaction if more than 1 house is concerned; (E), interaction providing estimate of error variance; NS, not statistically significant at the 0.05 level of probability; *, **, ***, respectively statistically significant at the 0.05, 0.01, and 0.001 levels of probability.

cluding shipments of lemons and Valencia and navel oranges. Experiments 1–6 include only naturally inoculated fruit in 5–8 replicated cartons per treatment. Experiments 7, 8, and 9 contain 3–5 replications per treatment, with each test carton containing 25 wounded and inoculated fruits distributed throughout the carton.

The statistical design was a simple randomized block type in a number of cases, and a split-plot type in the remaining experiments. The appropriate partitioning of degrees of freedom and sum of squares was made in each case. Tables by Duncan (1) were used to make the multiple range tests of mean differences.

Two or three treatments and a check were tested in each experiment. The treatments consisted of biphenyl sheets and ammonia tablets in all cases; where used, the third treatment was ammonia sheets.

Observations of the uninoculated fruit were made on the number of fruit per carton responding × 100 divided by the total number of fruit per carton for decay, soilage, and black buttons. For inoculated fruit, the number decayed out of 25 gave the per cent decay. The percentages were transformed to angles in degrees (angle = arcsin √ percentage) for statistical analysis and evaluation. The means of the percentages are

Table 2.—The per cent decay, soilage, and black buttons for 6 experiments with uninjured uninoculated fruit.

Treatments	Expt. 1 12 cars navels		Expt. 2 11 trucks navels		Expt. 3 8 cars lemons		Expt. 4 8 cars lemons		Expt. 5 11 trucks lemons		Expt. 6 12 cars Valencias	
					% Decay							
	(1)[a]	(2)[a]	(1)	(2)	(1)	(2)	(1)	(2)	(1)	(2)	(1)	(2)
Check	a 8.56	a	a 2.72	a	a 2.53	a	a 1.65	a	a 0.91	a	a 2.56	a
Ammonia sheets	b 3.75	b	b 1.80	bc	– –	–	b 0.92	b	b 0.75	ab	ab 1.93	ab
Ammonia tablets	b 3.68	b	b 2.00	b	b 1.05	b	b 0.64	b	bc 0.46	bc	ab 1.93	ab
Biphenyl sheets	b 2.86	b	c 1.48	c	c 0.35	c	b 0.69	b	c 0.32	c	b 1.57	b
					% Soilage							
	(1)	(2)	(1)	(2)	(1)	(2)	(1)	(2)	(1)	(2)	(1)	(2)
Check	a 5.5	a	a 4.4	a	a 6.0	a	a 7.7	a	a 3.5	a	a 4.4	a
Ammonia sheets	ab 4.2	ab	ab 3.0	ab	– –	–	a 5.5	a	ab 2.7	ab	a 4.0	a
Ammonia tablets	bc 2.4	b	b 2.0	b	b 2.6	b	a 3.9	a	b 2.1	b	a 4.0	ab
Biphenyl sheets	c 1.4	b	c 1.2	c	c 0.3	c	b 0.5	a	c 0.2	c	b 2.0	b
					% Black Buttons							
	(1)	(2)			(1)	(2)	(1)	(2)			(1)	(2)
Check	ab 11.5	ab			ab 2.0	ab	ab 13.3	ab			b 6.3	b
Ammonia sheets	a 13.2	a	[b]		– –	–	a 14.2	ab	[b]		a 19.2	a
Ammonia tablets	ab 11.3	ab			a 2.6	a	a 17.4	a			a 15.6	a
Biphenyl sheets	b 9.8	ab			b 1.4	b	b 11.2	b			b 7.8	b

[a] The letters a, b, and c before and after mean values indicate statistical populations at the 95% (1) and 99% (2) confidence levels.

[b] House × treatment interactions, see Fig. 2.

tabulated. Coding symbols used in this paper for the various sources of variation are shown in the footnote of Table 1.

Results.—*Uninjured uninoculated citrus.*—1) *Decay.*—The assignable sources of variation and statistical significances for 6 experiments are shown in Table 1. In Experiment 3, T × S(H), indicated in the table by (E), provided the estimate of error variance. In the other cases this estimate was obtained by replicating cartons within the classifications shown in the table.

The house mean square tested by S(H) was significant in the Valencia experiment (No. 6). Four houses were concerned, and a graph of the differences in decay among them is shown in Fig. 1. The per cent decay was about 8 times as high in House 6 as in each of the other 3 houses.

Shipments showed statistically significant differences at at least the 0.01 level of probability.

Treatment differences were statistically significant at the 0.001 level in 5 of the 6 experiments. In the Valencia experiment (No. 6) T × S(H) was significant at the 0.05 level, and was used to test treatment differences. The treatment mean square was significant at the 0.05 level only.

T × H and T × S(H) were not significant elsewhere.

In Table 2 the treatment means and check mean are given as per cent decay, with the statistical populations indicated to the left and right of the means. The confidence levels (C.L.) are given as 95% and 99%, although the joint confidence levels are somewhat lower.

The incidence of decay in the check was consistently higher than that in the ammonia or biphenyl treatments, and was statistically separable from its closest neighbor in 5 of the 6 experiments (95% C.L.).

Where the means of ammonia sheets, ammonia tablets, and biphenyl were statistically separable, biphenyl-treated fruit had the least decay.

2) *Soilage.*—The assignable sources of variation and significance for the amount of soilage are shown in Table 1. Where T × S(H) was used as the estimate of error variance, the entry in the body of the table is shown as (E).

House differences were significant at the 0.05 level in Experiment 6 (Fig. 1). The mean of House 6 is significantly different from those of the remaining houses.

Shipment differences were significant at at least the 0.05 level in all 6 experiments.

T × H was not significant in the 5 experiments in which the effect could be tested, and T × S(H) was not significant in the 2 experiments in which the effect could be tested.

Treatment differences were significant (0.05–0.001 level) in all 6 experiments (Table 2). The check

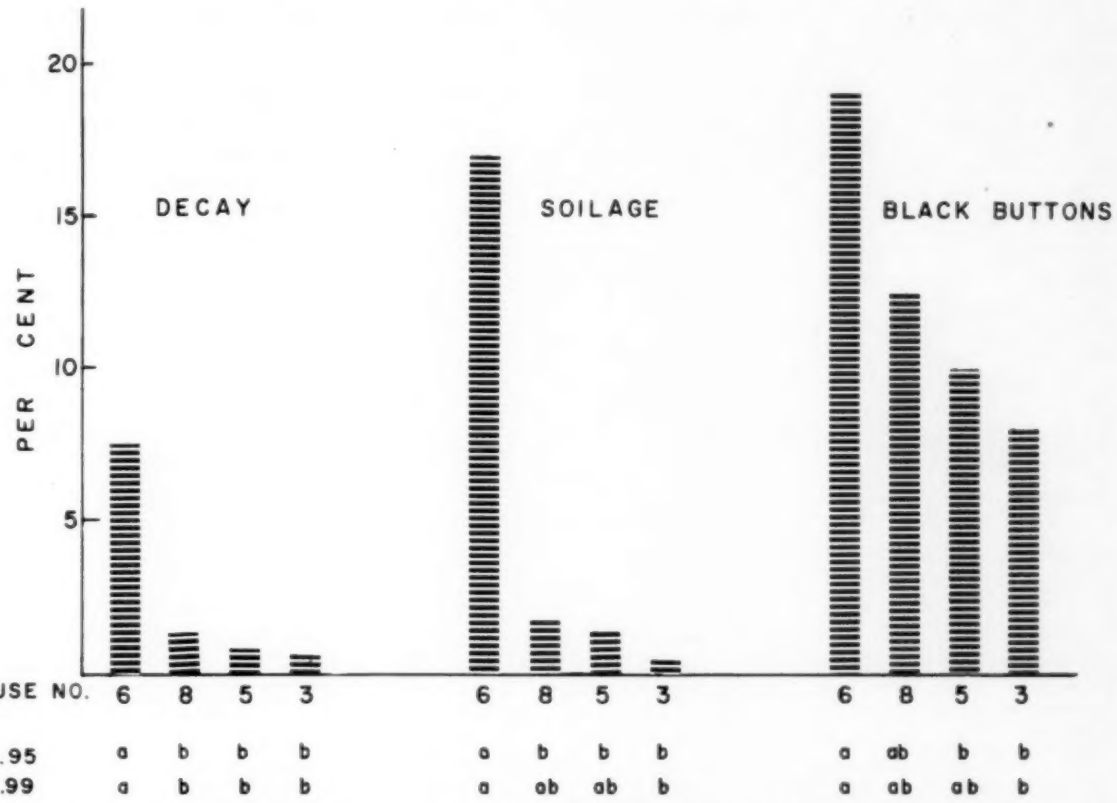

Fig. 1.—Analysis of 4 houses in Experiment 6 (Valencia oranges), showing variation between houses within a given experiment.

[1]The letters a and b indicate statistical populations at the 95% and 99% confidence levels.

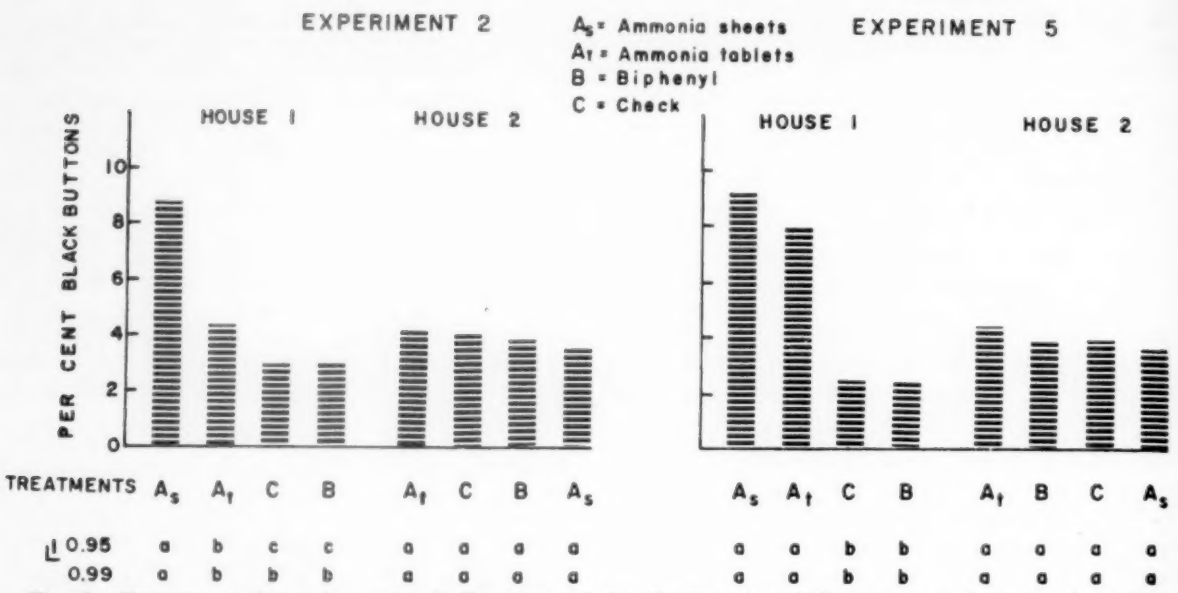
EXPERIMENT 2
A_s = Ammonia sheets
A_t = Ammonia tablets
B = Biphenyl
C = Check
EXPERIMENT 5
HOUSE 1
HOUSE 2
HOUSE 1
HOUSE 2
PER CENT BLACK BUTTONS
0
2
4
6
8
10
TREATMENTS A_s A_t C B A_t C B A_s A_s A_t C B A_t B C A_s
0.95 a b c c a a a a a a b b a a a a
0.99 a b b b a a a a a a b b a a a a

Fig. 2.—Treatment × house interaction in Experiment 2 (navel oranges) and Experiment 5 (lemons), showing increased black button injury by ammonia in two houses and not in two others.

[1]The letters a, b, and c indicate statistical populations at the 95% and 99% confidence levels.

mean was consistently higher than each of the treatment means in all cases, but statistically different (95% C.L.) from its closest neighbor in Experiment 3 only. The biphenyl mean was consistently low, and was statistically different (95% C.L.) from either ammonia mean in 5 of the 6 experiments.

3) *Black buttons.*—Table 1 shows the assignable sources of variation and statistical significances.

House differences were significant only in the Valencia experiment (No. 6) at the 0.05 level (Fig. 1).

Shipment differences were significant at at least the 0.01 level in 5 of the 6 experiments.

Treatment differences were significant in Experiments 1, 3, 4, and 6. In the remaining 2 experiments, T × H was significant and was used to test T. A multiple range test of T in each house in these 2 experiments showed that there were significant differences in one of the 2 houses in both cases. T × H was not significant elsewhere. The treatment means and statistical populations are given in Table 2, and T × H interaction is shown in Fig. 2.

Decay in injured inoculated fruit.—The results obtained from 5 shipments of navel oranges, 12 shipments of Valencia oranges, and 22 shipments of lemons are shown as Experiments 7, 8, and 9 in Table 3.

Shipment differences and treatment differences were highly significant in all 3 experiments. Since almost all lemon fruits in the check treatment of Experiment 9 were decayed, only the ammonia- and biphenyl-treated fruits were analyzed statistically. The check means were significantly different from the treatment means in the 3 experiments. The treatment means, however, did not follow a consistent pattern; in navels the means for ammonia and biphenyl could not be statistically separated; in the remaining 2 experiments with Valencias and lemons they were separable, biphenyl having a smaller mean than ammonia in lemons; the reverse was found with Valencias.

T × S was significant at the 0.001 level in the 2 experiments in which it could be tested.

Table 3.—Source of variation and per cent decay for 3 experiments with wounded inoculated fruit.

Source of variation[a]	Expt. 7 5 trucks navels			Expt. 8 12 cars Valencias			Expt. 9 22 cars lemons		
S		***			***			**	
T		***			**			***	
T × S		***			***			(E)	
Treatments	(1)[b]		(2)[b]	(1)		(2)	(1)		(2)
Check	a	54.8	a	a	68.2	a	a	98.8	a
Ammonia sheets	b	10.1	b	–	–	–	–	–	–
Ammonia tablets	b	10.8	b	c	25.8	c	b	51.9	b
Biphenyl sheets	b	25.2	b	b	40.1	b	c	33.8	c

[a] *Code symbols:* S = shipments, T = treatments, T × S = treatment by shipment interaction; **, *** = statistical significance at the 0.01 and 0.001 levels of probability, respectively; (E) = interaction providing estimate of error variance.

[b] The letters a, b, and c before and after mean values indicate statistical populations at the 95% (1) and 99% (2) confidence levels.

Table 4.—Injury to rind of citrus by ammonia.

Expt. no.	Variety	County shipped from	Cars (No.)	% of fruit injured					
				Ammonia tablets			Ammonia sheets		
				Slight	Moderate	Severe	Slight	Moderate	Severe
1	Navel	Tulare	1–7	0	0	0	0	0	0
1	Navel	Tulare	8–23	0.3	0.7	0.8	2.8	1.8	1.5
2	Navel	Ventura	1–2	0.7	2.0	0	2.5	10.0	0.3
2	Navel	Riverside	3–11	0.8	0.3	0.4	1.5	0.4	0
3	Lemon	Ventura	1–23	0	0	0	0	0	0
4	Lemon	Tulare	10–17	1.0	0.4	0.5	2.1	0.9	0.9
5	Lemon	Ventura	1–2	1.0	0.3	0	1.0	0	0
5	Lemon	San Bernardino	3–11	0.6	0	0	1.0	0	0
6	Valencia	Orange	1–41	0	0	0	0	0	0

1) *Ammonia injury.*—Fruit blemishes typical of ammonia injury were not found on any of the untreated fruits or on those exposed to biphenyl sheets. Injury ranging from slight to severe, however, was recorded for the ammonia treatment in some shipments and was absent in others (Table 4). Injury was in the form of red to brown sunken spots, and was generally noted on those fruits in direct contact with sheets or tablets. Where injury occurred, it was associated with the condition that allowed cool fruit to warm up in a humid atmosphere, thereby releasing an excessive quantity of ammonia from the sheets or tablets thus moistened by the sweating fruit.

Discussion.—The primary objective was to evaluate ammonia as a substitute for biphenyl. The results clearly demonstrate that ammonia, though significantly better than no treatment, was generally not as effective as biphenyl for control of decay and reduction of soilage in the uninjured uninoculated shipments. Ammonia-treated fruits also had a higher percentage of blackened buttons and surface injury than those treated with biphenyl.

The ammonia-generating sheets caused severe injury to some citrus fruit shipped from Tulare or Ventura County, California. This was much less evident in shipments from Riverside or Orange County. The reason may be the sudden condensation of moisture, wetting the chemicals and causing rapid generation of large quantities of ammonia over a short period. Also, citrus fruit from central California (where most of the injury occurred) is more susceptible to rind injury. Ammonia injury was rare in the many experimental tests with local Riverside fruit. No injury was observed on Valencia oranges in any of 41 cars shipped.

There appears to be an incongruity in the performance of a treatment or treatments, depending on the method of inoculation used. In Experiment 8 in Table 3, ammonia treatment was superior to the biphenyl and check treatments in control of decay of artificially wounded and inoculated Valencia oranges. These results are in agreement with laboratory results at Riverside, where, over a 3-year period, decay in artificially wounded and inoculated Valencia oranges treated with ammonia, biphenyl, and no treatment respectively averaged 13, 37, and 80%. Similar tests locally with navel oranges respectively averaged 21, 53, and 91% decay. Note, however, with uninoculated Valencia oranges (Table 2, Experiment 6) the ammonia treatment could not be separated statistically from that of biphenyl, and in Experiment 2 (Table 2) biphenyl was significantly superior to ammonia in control of decay with navel oranges. Reasons for this incongruity may be speculated on as follows: 1) Under commercial conditions, time of infection of fruit and period of incubation are not known and not controlled. Fungus spores in a wound in the fruit peel may remain ungerminated because of unfavorable temperatures and/or lack of moisture, or they may have germinated but their germ tubes have not yet invaded the rind, or the germ tubes may have begun to penetrate the rind tissues without yet producing macroscopic evidence of decay. In the first and second conditions, vapors of ammonia or biphenyl, if in adequate concentration, can inhibit decay. In the third condition, the lethal or inhibitory properties of the chemicals may or may not be effective, depending on the depth of penetration of the germ tubes. 2) The quantity of spores involved in the two methods of inoculation may be a factor in the subsequent effectiveness of a treatment. The high spore population in an artificial wound (2000 to 20,000 spores per wound) would provide a number of germinating and penetrating spores, some of which would resist or escape the fungistatic action of the biphenyl vapor. Where only a few spores reach a wound, however, as under conditions of natural inoculation, the probability of the occurrence of spores resistant to or escaping the biphenyl vapor would be much reduced.

The use of refrigerator cars or truck shipments with uninjured uninoculated fruit has the advantage of duplicating the actual conditions under which fruit is shipped, and in an emergency may be the only way a new treatment can be evaluated. There are certain inadequacies, however, that should be considered in such a method of evaluation. For example, the incidence of decay in California shipments is highly variable with some shipments having little or no decay, thus increasing the number of tests necessary to obtain reliable data. This is both expensive and time-consuming. Uncontrolled variations in fruit handling, in packing-house sanitation, in the vitality of various

lots of fruit, in the characteristics of different refrigerator cars, in methods of fruit cooling, etc., make valid comparisons of treatments difficult to obtain. This was shown in the significant house differences (Experiment 6, Table 1, and Fig. 1), treatment by house interactions (Fig. 2), and the degree of variability in shipments within houses or between houses (Table 1). Future test shipments should consider the placing of these variables under control wherever possible if more reliable data with fewer shipments are to be expected.—Department of Plant Pathology and Biometrical Laboratory, University of California Citrus Experiment Station, Riverside.

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A SOIL INFESTATION METHOD FOR STUDYING SPORES OF HELMINTHOSPORIUM SATIVUM

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SUMMARY

A soil infestation method was designed for studying conidiospores of *Helminthosporium sativum* Pam., King, & Bakke in soil. By this method, the conidiospores are deposited directly in soil in a manner whereby they can be quickly found and easily removed from the infested soil even after 8 months' incubation. Results from studies with this soil infestation method showed that less than 1% of the conidiospores of *H. sativum* germinated in natural soil at 25° and 3°C. In sterilized soil, however, 10% and 24% of the spores germinated at 25° and 3°C, respectively. Maximum spore germination occurred in 2 days in sterilized soil maintained at 25°C, whereas maximum germination at 3°C required 8 days. Chinn's glass-slide technique yielded results similar to those obtained with the soil infestation method in natural soil. However, in sterilized soil, using the same technique, more than three times as many spores germinated at 25° and 3°C. The results from studies on spore germination suggest that the soil infestation method is more sensitive to the soil fungistatic factor, which is purported to inhibit spore germination, than Chinn's glass-slide technique.

Introduction.—Symptoms of root rot of wheat incited by *Helminthosporium sativum* Pam., King & Bakke on winter wheat grown in western Nebraska during the past 4 years were first detected seasonally in late November and early December. Dark brown lesions were present in varying degrees on all parts of the roots and on the subcrown internode of the early- and late-planted wheat; disease incidence was usually highest in early plantings. Soil temperatures at the time of incipient symptom development were frequently near freezing for protracted periods. These findings were unexpected, for it has generally been assumed that the symptoms of this malady on winter wheat are manifested in early fall and in the spring and summer, when soil temperature and other extrinsic environmental factors are apt to favor infection. It is apparent from the results of the field surveys that considerably more information is needed on the epidemiology and ecology of *H. sativum* before better methods of control can be developed.

Previous research on root diseases of cereals incited by *H. sativum* indicated that the pathogen persists in soil as conidiospores (6, 8). It follows that the first sequence of events leading to penetration and subsequent infection of the roots of susceptible hosts is concerned with germination of the conidiospores. Much work has been done to delineate factors affecting spore germination of *H. sativum* in soil, but many phases of the problem need to be resolved.

The principal difficulty with studies of fungus spores in soil is the lack of adequate methods and techniques. The ingenious glass-slide technique recently devised by Chinn (1) for studying spore germination in soil has been instrumental in advancing our knowledge in this refractory area of soil microbiology. There are, however, several inadequacies in Chinn's method for

studying fungus spores in soil. The use of glass slides coated with films of water agar for the deposition of spores may provide an environment vastly different from that in soil *per se*. Also, the glass slides tend to separate from sandy loam soils maintained at low soil moisture. The resulting space between the glass slides and soil could drastically alter the concentration of the fungistatic materials as well as upset aeration and humidity in the immediate vicinity of the spores. This paper describes a new method for studying *H. sativum* in soil without employing foreign materials for the deposition of spores.

Materials and methods.—Composite samples of a fine sandy loam soil from the top 4-in. layer were collected from a commercial wheat field in western Nebraska in August, 1958. All samples of soil were thoroughly mixed and subsequently sieved through a 0.5-mm screen. The processed soil was then transferred to galvanized 50-gal. cans and stored at 15°C.

To assure uniform distribution of water in soil and to prevent excessive packing of soil in pots, the following procedure was adopted. New 5-in. pots were filled with 930 g of soil, which was then slowly wetted to field capacity. For the sterilized soil treatment, the potted soil was autoclaved at 15 lb pressure for 1.5 hr on each of two consecutive days. In the unsterilized soil treatment, the pots were incubated for about 24 hr at 25°C before proceeding further. Next, in both treatments, the soil was removed from each pot, mixed separately, and then returned to the same pot. Care was taken to minimize contamination of the sterilized soil treatment. Each pot was filled with the re-worked soil to a level 2¼ in. from the top. The surface of the soil in each pot was made smooth by gently tapping it with a Petri plate cover (Fig. 1-A). Care was taken to avoid excessive packing of the soil in this operation.

The smooth surface of soil was then infested with conidiospores of *H. sativum*. The pathogenic isolate used in this study was derived from a single spore originally obtained from diseased roots of winter wheat. This isolate was strongly pathogenic in causing root rot of wheat grown in sterilized and un-

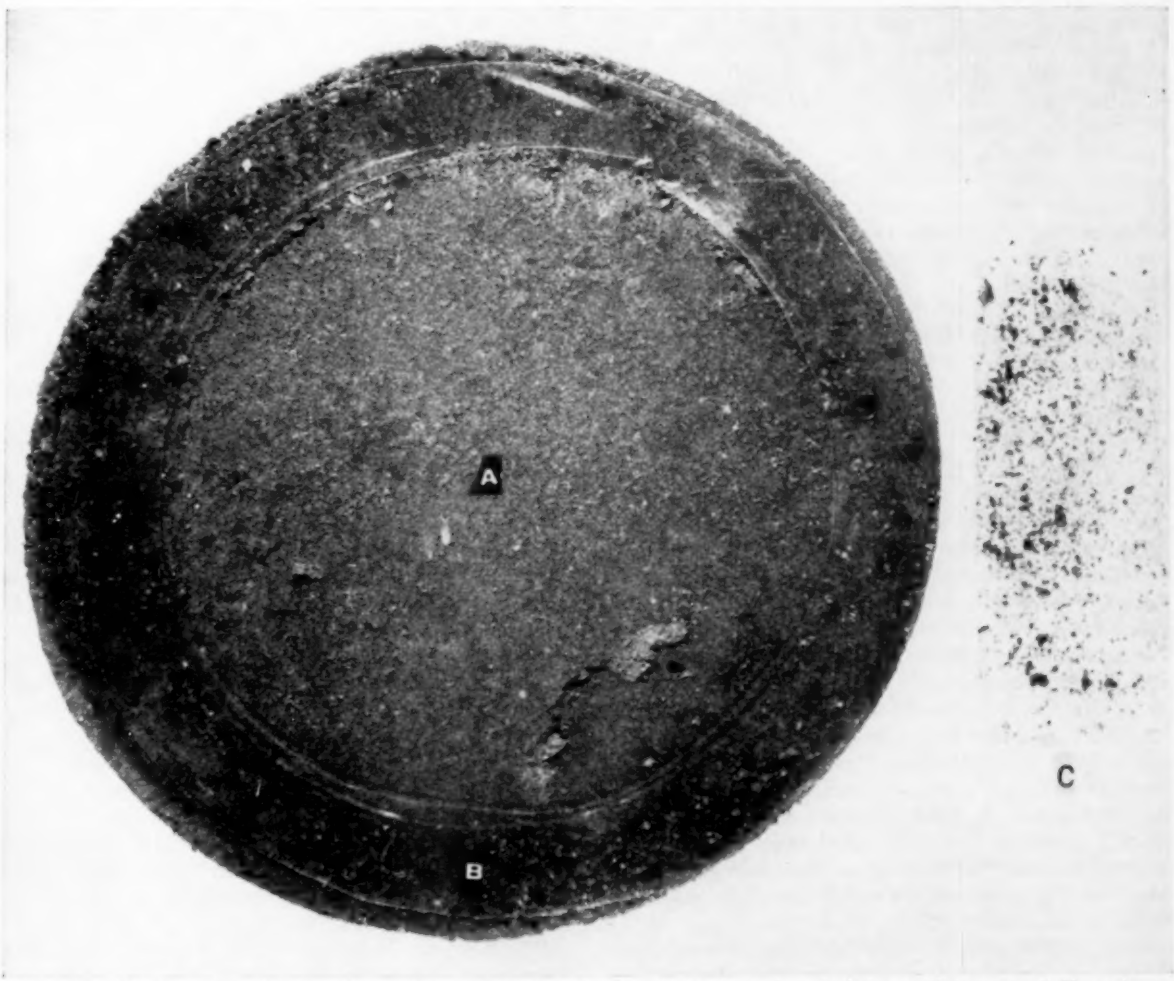

Fig. 1.—**A)** Top view of soil column with smoothed surface ready to be infested with conidiospores of *H. sativum*. **B)** Ring-shaped membrane used as marker to locate soil plane infested with spores. **C)** Block of water agar used to make spore print from infested soil.

Table 1.—Effect of soil treatment on conidiospore germination of *Helminthosporium sativum* as determined by the soil infestation method and Chinn's glass-slide technique.

Treatment		% germination[a] at various incubation intervals (days)							
		1	2	3	5	6	8	10	12
Soil infestation method									
1. Natural soil	25°C	0	0	0	<1	0	0	<1	<1
2. Natural soil	3°C	1	0.5	0	<1	<1	<1	1	<1
3. Sterilized soil	25°C	8	10	10	6	4	5	2.5	2
4. Sterilized soil	3°C	0	4	15	18	21	24	17	10
Chinn's glass slide									
5. Natural soil	25°C	0	0	0	0	0	0	<1	0
6. Natural soil	3°C	0	0	0	<1	0	<1	0	0
7. Sterilized soil	25°C	51.5	53	55	58				
8. Sterilized soil	3°C	1	17	47	69				

[a] Per cent germination is based on 2000 spores from two pots for the soil infestation method, and on 1800 spores on 6 slides from two pots for Chinn's glass-slide technique.

sterilized soil in the greenhouse. The medium for maintaining the pathogen and for increasing inoculum was prepared by adding 25 ml of tap water to each 250-ml Erlenmeyer flask containing 10 g of washed roots excised from 3-week-old Cheyenne wheat grown in the greenhouse. This root-medium was sterilized for 25 min at 15 lb pressure and then infested with a 10-day-old culture of the pathogen maintained on this medium. After the cultures were incubated for 30 days, the excess water in each flask was discarded and replaced with about 150 ml of tap water. The flask was then vigorously shaken and the water suspension of conidiospores was strained through a double layer of cheesecloth. The conidiospores from one flask were sprayed on the smooth soil surfaces of 16 pots with a DeVilbiss atomizer attached to an air line regulated to 8 lb pressure. It was estimated that about 60,000 conidiospores were deposited on the soil surface of each pot. Viability of the inoculum was determined by spraying 10 aliquots from the water suspension of conidiospores into Petri plates containing potato-dextrose agar (PDA, 1% dextrose, pH 6.5) and incubating at 25°C for 6 hr. The surface of the PDA was then sprayed with lacto-phenol cotton blue to facilitate observation of germ tubes. The percentage of spore germination was based on 1000 spores, representing a random examination of 200 spores in each of 5 Petri plates. It was interesting to note that the viability of the conidiospores from 30-day-old cultures grown on the root-medium was never less than 94% in any of the tests in this study. On the other hand, the viability of spores from 30-day-old cultures on PDA and on sterilized seed of wheat supplemented with water was about 80% and 60%, respectively. It was also noted that the pathogen produced fewer sectors on the root-medium than on PDA. Results from preliminary studies indicate that this particular isolate of *H. sativum* was most pathogenic to wheat when the inoculum was obtained from root-medium rather than from PDA or sterilized wheat.

Immediately after the smooth surface of soil was infested with conidiospores, a ring-shaped membrane of Polyflex 230 with a thickness of 0.13 mm was placed on top of the soil. This membrane was 4 in. in diameter, with a 0.5-in.-wide rim and an opening 3.0 in. in diameter (Fig. 1-B). The membrane had been washed in water to which detergent had been added. This solution containing the membrane was then heated for 2 hr to 75°C to remove toxic materials. Other materials such as cellulose nitrate and cellulose acetate may be substituted for Polyflex 230. Results from preliminary experiments indicated that none of these materials inhibit germination of conidiospores of *H. sativum*. The infested surface layer of soil and the ring-shaped membrane were covered with the remainder of the soil from each pot. The top layer of soil over the ring-membrane was about 1.5 in. deep and extended to about ¾ in. from the top of the pot. This narrow, circular band of Polyflex separated only the peripheral area of the two layers of soil. The major portion of the surface layer of soil infested with spores, therefore, was in immediate contact with soil of the upper layer. The membrane was employed merely to facilitate location and separation of the soil-plane infested with conidiospores.

Half of the pots containing the infested unsterilized soil or the sterilized soil were incubated at 3°C, and the remainder at 25°C. Soil moisture was regulated to about 50% of its moisture-holding capacity under ⅓ atmosphere tension.

At various intervals (Table 1) for 12 days, 2 pots from each of the 4 treatments were sacrificed in making determinations of spore germination and viability.

The removal of an intact column of soil from pots is a requisite for locating the layer of soil infested with conidiospores. Because excellent tilth of the soil caused it to fragment very easily during removal from the pot, the soil column in the pot was first compressed. To do this, a metal plate about 4 in. in diameter was placed on top of the soil in each pot and firmly pressed down until the soil did not yield to pressure. Watering the soil to field capacity 2 days prior to the above operation was extremely helpful in maintaining the soil column intact.

The soil was removed from the pot by placing a Petri plate cover on the surface of the soil and then inverting the pot and gently tapping the rim until the soil column was free and resting on the Petri plate

cover. With a spatula, about 2 in. of soil was trimmed from the end of the soil column that was at the bottom of the pot. A second Petri plate cover was placed on the trimmed end of the soil column. The column of soil was then turned upright while the soil was held in place by gently pressing the glass covers against the ends of the soil column. The smooth plane of soil originally infested with conidiospores was readily located and exposed by carefully removing the top layer of soil from the buried disk membrane of Polyflex. With a little practice, the infested layer of soil can be uncovered by this procedure. Even 8 months after the conidiospores were embedded in the soil it was possible to separate the soil column at the plane originally infested with this pathogen. Studies now in progress indicate that it may be possible to locate the spores of *H. sativum* a year after the soil is infested.

Removal of the conidiospores from the soil for microscopic examination was accomplished without materially disturbing their relative position. Slabs of 1.5% water agar were used to determine the percentage of spore germination in soil. The slabs of water agar, 2¼ × 1 in., were cut from Petri plates containing 20 ml of the medium. A spore print was made by placing the strip of water agar on the infested soil and gently pressing it against the soil with the fingers (Fig. 1-C). Along with small soil particles, as many as 1500 spores adhered to the block of water agar. Microscopic examination of the spores was not seriously hampered by the soil particles. The slab of water agar was transferred to a standard-size microscope glass slide, stained with lacto-phenol cotton blue applied with an atomizer, and immediately examined for spore germination. Two spore prints were prepared from each of two pots for each treatment. A random sample of 500 spores was examined on each block of water agar, making a total of 1000 spores for each pot.

The above procedure was also employed with slabs of PDA to determine the viability of the ungerminated spores. Spore prints on this medium were incubated for about 6 hr at 25°C before staining.

The above method for studying conidiospore germination in soil was compared to Chinn's glass-slide technique (1), which was modified as follows. The procedure described previously for preparing soil for potting was also used for the glass-slide technique. Unlike Chinn's technique for infesting the glass slides, however, the conidiospores were deposited by means of a DeVilbiss atomizer on glass slides coated with 1% water agar, pH 6.5. Each slide contained about 1000 spores. Viability of the ungerminated spores was determined by placing the surface of spore-infested slides on PDA (pH 6.5) in Petri plates and incubating for 5 hr at 25°C. One slide from each pot was used to make viability determination. The remaining 3 slides in each pot were examined for spore germination that had occurred in soil. Studies with Chinn's glass-slide technique were run parallel to studies with the soil infestation method. Each experiment reported herein was repeated three times, with concordant results.

Experimental results.—*Studies with natural soil.*—Results from studies employing the soil infestation method and Chinn's glass-slide technique in studying *H. sativum* in natural soil are presented in Table 1. Germination of conidiospores as determined by the two methods was negligible in natural soil incubated at 25° and 3°C for 12 days. In part, these results are in accord with those reported by Chinn (1) for the spores of *H. sativum* incubated for 14 days in natural soil at "room temperature." The presence of some unknown material that inhibits spore germination, and referred to in the literature as soil fungistasis, may be responsible for the low percentage of spore germination in natural soil (2, 3, 4, 5, 7, 9). It has also been shown that nutrition may negate the fungistatic effect in stimulating spore germination of *H. sativum* in unsterilized soil (1, 3, 9). It is apparent, however, that both methods used to study spore germination are very sensitive to fungistatic and other soil factors that restrict spore germination in natural soil held at 25° and 3°C.

Although natural soil was not conducive to germination of conidiospores of *H. sativum*, it did not substantially reduce the viability of the spores in this study. At the start of the experiment, about 96% of the spores were viable. About 94% of the ungerminated conidiospores were still viable in natural soil kept for 19 days at 25° or 3°C. Results from subsequent studies, not reported here, indicate that over 80% of the spores were viable in natural soil maintained at 25° or 3°C for over 8 months. Very little difference was noted in viability of the spores in this study employing the soil infestation method or Chinn's glass-slide technique. Microscopic examination of conidiospores from supernumerary pots every 7 days did not reveal the presence of new spores of *H. sativum* during the incubation periods of 19 days or 8 months. These results lead to the conclusion that retention of viability by a high percentage of the spores during the short and long incubation periods in natural soil cannot be ascribed to the production of new crops of spores under the conditions of this investigation.

Studies with sterilized soil.—The soil infestation method yielded strikingly different results from Chinn's glass-slide technique in studies on the effect of soil sterilization and soil temperature on spore germination of *H. sativum*. The highest percentage of conidiospore germination, in either method, occurred in sterilized soil kept at 3°C (Table 1).

Chinn's glass-slide technique gave the higher percentage of spore germination in sterilized soil kept at 3°C. About 69% of the conidiospores of *H. sativum* had germinated on glass slides kept in soil for 5 days. Moreover, spore germination was uniformly distributed on the glass slides. Further observations on spore germination were not continued after 5 days' incubation, since extensive mycelial growth on the slides obscured many of the spores. At least 17% of the conidiospores had germinated on glass slides held in soil for 2 days. The length of the germ tubes varied from 20 to 90 μ, averaging about 60 μ. Nearly three times as many spores germinated in 3 days than in 2

days on glass slides buried in sterilized soil. The average length of the germ tubes of spores in soil for 3 days was about 200 μ. Lysis of a relatively few germ tubes and mycelia was discerned on glass slides in soil for 5 days. Over 93% of the ungerminated spores retained their viability on slides kept in soil 1–5 days.

Results from the glass-slide technique on spore germination in sterilized soil maintained at 3°C are also presented in Table 1. About 58% of the conidiospores germinated on glass slides kept at 25°C for 5 days as compared to 69% germination in a comparable test made at 3°C. In contrast to the small number of spores that germinated in 1 day at 3°C (1%), nearly 52% of the conidiospores germinated during this time at 25°C. During the subsequent 4-day incubation period, however, only 58% of the conidiospores germinated at 25°C. On the other hand, 69% of the spores germinated during this 4-day incubation in soil kept at 3°C. Over 93% of the ungerminated spores remained viable during the course of this experiment at 3°C.

With the soil infestation method, maximum spore germination occurred in about 8 days in sterilized soil kept at 3°C (Table 1). At this time, 24% of the spores produced germ tubes ranging from 20 to over 900 μ long, averaging about 700 μ. Germination of conidiospores began after 1 day incubation in soil, but only 4% of the spores had germinated in 2 days. The highest increase in spore germination in a 24-hour period of incubation occurred between the 2nd and 3rd days. During this time, spore germination quadrupled.

Further, the average length of the germ tubes was about 30 and 150 μ, respectively. Spore germination after 3 days of incubation increased slowly until the 8th day, when maximum germination was attained. Apparently, few spores germinated between the 8th and the 12th days of the incubation period. Plasmolysis of many germ tubes and mycelia was evident during this time. Failure of the plasmolyzed germ tubes to stain accounts for the fewer germinated spores detected after 8 days of incubation. Again, over 94% of the ungerminated spores retained their viability during the course of the experiment.

Germination of conidiospores of *H. sativum* in sterilized soil incubated at 25°C was relatively low in studies employing the soil infestation method (Table 1). Maximum germination of spores, 10%, was established within 2 days' incubation. The average length of the germ tubes during this period was 400μ compared to about 50μ at 3°C. Although the germination count did not increase during the subsequent 24 hours, the germ tubes attained an average length of about 600μ. Lysis of the germ tubes after an incubation period of 3 days accounts for the lower counts of germinated spores during the latter part of the experiment. Viability was retained by over 93% of the ungerminated spores during this study.

Discussion.—A new method was devised for infesting soil with conidiospores of *Helminthosporium sativum* without employing foreign materials on which to deposit spores. Thus the immediate vicinity of the soil infested with spores does not come in contact with materials, such as glass slides, which could upset the natural environment of the soil. With this new method, the spores can be easily located and readily recovered from soil even after 8 months. Furthermore, a large number of spores can be removed from the same relative position they occupied in the soil.

Several investigators have shown that the soil fungistatic factor(s) may be negated by sterilizing the soil with steam (1, 2). It is not known, however, whether the accompanying stimulation in spore germination following soil sterilization is due to the elimination or reduction of the fungistatic material and/or to the increase of nutritive materials or to some other factors. In this study, the highest percentage of spore germination (69%) was obtained with Chinn's glass-slide technique in sterilized soil at 3°C for 5 days. The same technique used in soil kept at 25°C yielded 10% fewer germinated spores than at 3°C. The higher number of germinated spores at 3°C may be related to the relatively slower rate by which microorganisms colonize sterilized soil maintained at near-freezing temperature. Consequently, a longer time would be required to accumulate a sufficient amount of the fungistatic substance to inhibit spore germination at 3°C than in soil at 25°C.

The above hypothesis is also applicable in explaining differences in the number of conidiospores germinating at high and low soil temperatures with the soil infestation method. Table 1 shows that twice as many spores germinated at 3°C as at 25°C. It is noteworthy that spore germination was maximum in 2 days in soil at 25°C, and in 8 days at 3°C.

It is difficult to explain why the soil infestation method yielded a substantially lower percentage of spore germination than Chinn's glass-slide technique at 25° and 3°C (Table 1). The soil infestation method is perhaps considerably more sensitive than Chinn's glass-slide technique to low concentrations of fungistatic substances in soil. The greater sensitivity of the former method might be due to the fact that the spores are in closer proximity to the soil than they are on glass slides buried in soil. Differences in spore germination in the two methods cannot be ascribed to the different amounts of inoculum used. In subsequent studies, not reported here, the difference in percentage of spore germination was just as great when an equal number of conidiospores was used in the 2 methods as when the inoculum in the soil infestation method was 60 times as great as that used in Chinn's glass-slide technique.

Germination of conidiospores of *H. sativum* in sterilized soil at 3°C provides some clues in explaining why symptoms of root rot of winter wheat grown in western Nebraska develop in late November and early December. It is reasonable to assume that the marked decline of the metabolic activities of microorganisms in natural soil at temperatures near freezing may result in a low concentration of the fungistatic materials. Such a concentration of the fungistatic factor, coupled

with the availability of nutritive materials in the rhizosphere of wheat, provides an environment that is conducive to spore germination. Further development of the germ tubes in the rhizosphere may culminate in infection and subsequent development of symptoms on roots of wheat in soil at temperatures near freezing.—Department of Plant Pathology, Nebraska Agricultural Experiment Station, Lincoln.

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PHYTOPATHOLOGICAL NOTES

EVIDENCE OF FUNGISTATIC ACTION OF PETROLEUM OIL AGAINST MYCOSPHAERELLA MUSICOLA INSIDE BANANA LEAVES

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The action of oil mist sprays in control of the Sigatoka leafspot disease of bananas is being studied. A previous report (2) stated that oil did not inhibit the pathogen, *Mycosphaerella musicola* Leach, either *in vitro* or on the leaf surface, and suggested that oil exerted its antifungal effect after the fungus penetrated the leaf. It was not known, however, whether the oil was acting as a fungicide or a fungistat. The present study was conducted to determine: 1) whether oil could control the disease when applied after the fungus penetrated the leaf, and 2) whether the fungus was dead or alive inside an oil-sprayed leaf.

The experimental procedure was to inoculate four 1-in.-sq areas on the lower leaf surface of the youngest unfurled leaf on 3 Dwarf Cavendish banana plants. A pathogenic strain of *M. musicola* was used that sporulates in culture and is easily distinguished from wild-type material because of morphological differences in colonial growth. The conidial suspension was applied with a camel's-hair brush. One week after inoculation, fungal penetration into the leaf was determined. One inoculated area was removed from each leaf, stained, and examined microscopically; 35–60% of the spores had growth that penetrated through the stomata.

After penetration was known to have occurred, one inoculated area and adjacent tissue on each leaf was sprayed with oil (Oil #LO-2144, viscosity 88.5 S.U.S. at 100°F, sp. gr. 0.85, unsulfonated residue 100%; obtained from Esso Research and Engineering Co., Linden, N. J.) by a precision technique described previously (1). The oil sprays were applied at the third, fifth, and seventh week after inoculation. The oil was deposited at the rate of 2.8 mg/100 sq. cm of leaf area, which is typical of the deposit resulting from a spray rate of 2 gal./A. (1). This spray procedure approximated commercial conditions where 1–3 gal./A. are misted on the plants at 2–4-week intervals.

Sigatoka spots were present on the remaining 2 inoculated unsprayed areas of each leaf 7 weeks after inoculation. Conidia from the diseased areas were isolated on agar, and colonies formed that were similar morphologically to those of the strain used for inoculum. The sprayed inoculated areas remained disease-free throughout the experiment.

Ten weeks after inoculation (7 weeks after the first spray), the sprayed inoculated area was detached from each of the 3 leaves and was surface-sterilized by agitating for 2 minutes in an aqueous solution containing 1% $HgCl_2$ and 0.25% Triton X-114. (Wetting agent added to insure thorough wetting of the waxy leaf surface. Product of Rohm & Haas Co., Philadelphia 5, Pa.) Preliminary tests showed that this treatment will kill conidia and germ tubes of *M. musicola* on the banana leaf surface. The leaf piece was then rinsed briefly in sterile water, homogenized for 1 minute in a Waring blender containing 100 ml sterile water, and 0.5-ml portions of the resulting suspension were plated on water agar. A series of 5 plates was prepared for each replicate leaf piece.

After 5 days at room temperature, each series of water-agar plates contained minute mycelial colonies, 36 of which were transferred to nutrient agar. All isolates developed into colonies that were morphologically similar to those of the strain used for inoculum. Spores from these colonies were used to inoculate banana leaves, and within 7 weeks typical Sigatoka disease symptoms appeared. This entire test was repeated, with similar results.

The following conclusions can be drawn from these trials: 1) The oil controlled the disease after the pathogen had already entered the leaf. 2) After 7 weeks inside an oil-treated leaf, at least some of the fungus was still alive and inherently pathogenic.

Oils vary greatly in their chemical and physical properties. It is possible that some oils might kill *M. musicola;* however, the present study suggests that the oil used controlled Sigatoka by a fungistatic action.—Federal Experiment Station, Agricultural Research Service, U. S. Department of Agriculture, Mayaguez, Puerto Rico.

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CONTROL OF THE MINT RUST FUNGUS, PUCCINIA MENTHAE, BY NICKEL SALTS

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When mint rust appears in epidemic form, both the leaf and oil yields are dramatically reduced. Resistance breeding has not produced commercially available resistant varieties, and no satisfactory control measures have been developed for the chemical protective or eradicative control of the disease. Recent successes with nickel salts against cereal and sunflower rusts (1, 3) warranted this study of the effect of $NiCl_2$ and $Ni(NO_3)_2$ on the development of mint rust.

$NiCl_2$ and $Ni(NO_3)_2$ solutions (0.1, 0.2, and 0.3%) were sprayed on *Mentha piperita* plots (180 m^2 each) at the rate of 1 L/10 m^2. A single spray was applied between the first and second mowings (early September), when the rust started to appear. The protective effect of nickel salts is indicated in Table 1. The data presented are based on the examination of 200–300 leaves detached at random in each plot 3 weeks after spraying. As may be seen, $Ni(NO_3)_2$ proved to be somewhat more effective than the other chemical.

Table 1.—Fungicidal activity of nickel salts against mint rust.

Chemical	% concentration	% occurrence of various infection classes[a]					
		0	1	2	3	4	5
$Ni(NO_3)_2$	0.1	35.5	42.5	13.5	7.5	1.0	0.0
	0.2	31.0	64.0	5.0	0.0	0.0	0.0
	0.3	55.5	43.0	1.5	0.0	0.0	0.0
$NiCl_2$	0.1	17.5	26.5	36.0	17.0	2.0	1.0
	0.2	27.0	46.0	23.0	3.5	0.5	0.0
	0.3	42.0	51.0	6.5	0.5	0.0	0.0
Control		19.5	21.5	16.5	12.5	18.0	12.0

[a] infection classes. 0 = no infection, 1 = 5 pustules per leaf, 2 = ¼ of the leaf surface covered with pustules, 3 = ½ of the leaf surface, 4 = ¾ of the leaf surface, 5 = the whole leaf surface covered with pustules.

Table 2.—Effect of control of mint rust by nickel salts on the oil yield.

Treatment	Concentration (%)	Oil[a] (ml)	Oil %
$Ni(NO_3)_2$	0.1	49	0.29
	0.2	25	0.15
	0.3	34	0.21
$NiCl_2$	0.1	30	0.18
	0.2	47	0.28
	0.3	19	0.11
None	—	18	0.11

[a] In 15 kg leaves.

The oil content of the samples has also been assayed by steam distillation, 15 kg of leaves being used for each determination. The data (Table 2) indicate a pronounced increase in oil content in all treated samples over the control.

Determination of the menthol content of the oil by a colorimetric procedure (2) gave on the average the following values: control, 62.5%; $NiCl_2$-treated, 63.5%; $Ni(NO_3)_2$-treated, 54.5%. Apparently the menthol content is reduced in the $Ni(NO_3)_2$-treated leaves.

The nickel residue in the leaves treated with the highest concentrations of nickel salts has also been assayed, using a colorimetric method based on the color formation of Ni in the presence of dimethylglyoxim. Respective findings were 5.7 mg% and 5.4 mg% (fresh weight) in the $Ni(NO_3)_2$- and $NiCl_2$-treated samples. The oil is free of nickel contaminations.

On the basis of the above results, nickel salts may be regarded as a valuable tool for the control of mint rust when mint is grown for oil production.—Research Institute for Plant Protection, Budapest, and Research Institute for Medicinal Plants, Budapest, Hungary.

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HETERODERA TRIFOLII, A FOLIAGE PATHOGEN OF WHITE CLOVER

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The genus *Heterodera* has long been recognized as being composed of nematodes parasitic on the roots and underground stems of vascular plants. Little attention appears to have been given to the possibility that these nematodes attack and reproduce in aboveground plant parts.

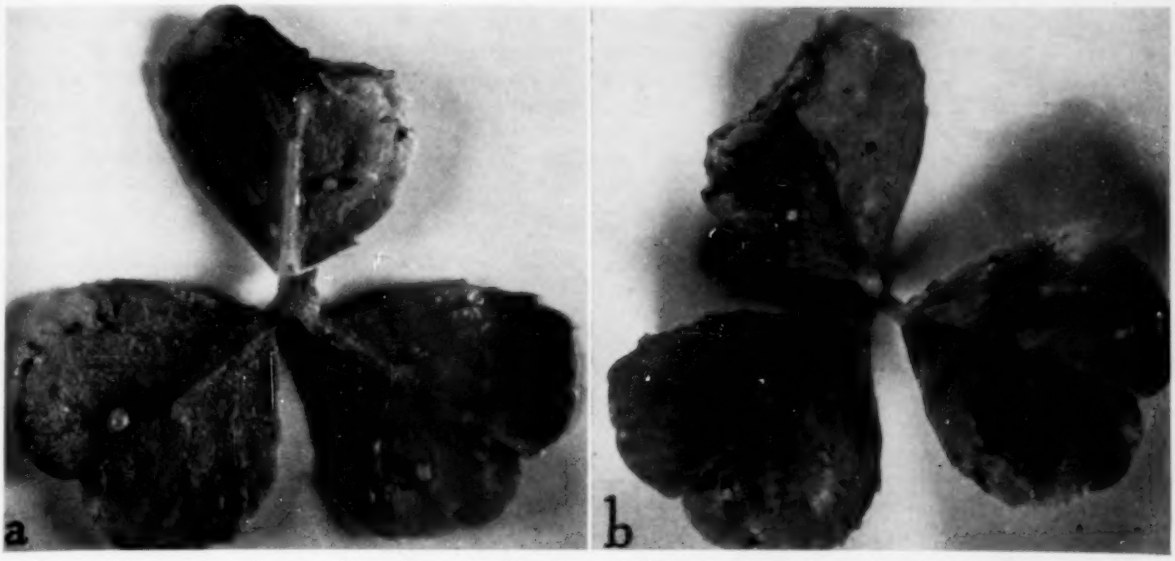

Fig. 1.—Lower (a) and upper (b) surfaces of white clover leaf showing developing white females and brown cysts of *Heterodera trifolii* (X5).

Gerdemann and Linford (2) mentioned that larvae of *Heterodera trifolii* Goffart, 1932, are capable of entering clover leaves covered with moist soil. They did not mention the fate of these nematodes.

In a recent greenhouse experiment with the cyst nematode of clover, *H. trifolii*, immature and mature females and brown cysts were observed on both surfaces of white clover leaves. A small leaf near the crown of the plant was heavily infected (Fig. 1), and leaves about 2 in. above the stolon also contained a few developing females. The plant was washed free of soil and kept moist for several days without soil in contact with it. When the leaves were stained with acid-fuchsin–lactophenol, larvae in various stages of development were visible within the leaf tissue. Many second-stage larvae were observed to invade the small heavily infected leaf; some had only their anterior ends in leaf tissue, whereas others appeared to be wholly within the leaf. These larvae had probably emerged from the brown cysts mentioned earlier since there was no other source of larvae. These observations indicate that larvae of *H. trifolii* are capable of invading leaves in the absence of soil.

Peculiar craters were observed in the leaf surface, presumably where cysts had fallen off. A small area of slightly swollen translucent leaf tissue near the anterior end of each female indicated that in leaves, as in roots, giant cells and hypertropy are induced by the feeding of the nematode.

The creeping growth habit of white clover permits leaves to originate near the soil, and in many instances the foliage remains moist for long periods. Both of these conditions would favor foliage infection in the field.

The clover cyst nematode is known to be widespread in Europe, Great Britain, and North America (1). Although Raski and Hart (3) said that new infestations of the clover cyst nematode in California appeared not to have originated from cysts mixed with clover seed, it is possible that cysts on leaves could be dislodged during the seed harvest and find their way into seed lots. The cysts are slightly smaller than the seed (0.5–0.6 mm vs. about 1 mm), but it is conceivable that enough cysts to establish new infestations could remain in seed harvested from infected plants. Gerdemann and Linford (2) reported that the brown cysts of *H. trifolii* are drought-tolerant and that larvae emerged from cysts after being stored 11 months in dry soil. The observation reported here may be relevant to dissemination of this nematode.—Department of Plant Pathology, North Carolina State College, Raleigh.

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CONIDIAL PRODUCTION FROM FILTER PAPER CULTURES OF HELMINTHOSPORIUM VAGANS AND ALTERNARIA SOLANI

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Conidia of uniform size and age for inoculum are difficult to obtain from *Helminthosporium vagans* and *Alternaria solani*. This note describes a simple method. The fungi are grown aseptically on a shaker in a liquid nutrient (1) for several days and then harvested. From this point on, aseptic techniques are not used. The mycelial pellets are fragmented with a

Waring blender for 2 minutes. The hyphal fragments are centrifuged, resuspended in 0.02*M* phosphate buffer (pH 6.4), and distributed in 2-ml portions on dry filter paper in Petri dishes. No nutrient is added. Cultures of *H. vagans* so prepared produce overnight at room temperature 1 conidium per conidiophore and, in 3 days, 3–5 conidia per conidiophore. Light has little influence on sporulation of *H. vagans*. In 24 hours under fluorescent light, *A. solani* produces conidiophores on filter paper. When these cultures are held 24 hours in either darkness or daylight, 50–75% of the conidiophores produce a conidium. Cultures of *A. solani* in continuous light produce no conidia, even after 7 days. Witsch and Wagner (2) reported similar light effects for *A. brassicae* var. *dauci*.

Washing of mycelium was necessary for conidial production by *H. vagans*. Hyphal suspensions after being ground and adjusted to pH 6 produce no conidia, whereas the same suspensions with supernatant removed and resuspended in phosphate buffer produced conidia in abundance. Washing mycelium has a less pronounced effect with *A. solani*.

This method of producing spores does not affect pathogenicity. Thus, conidia of *H. vagans* produced in 3 days on filter paper were pathogenic to *Poa pratensis*, and conidia of *A. solani*, similarly produced in 3 days, were pathogenic to *Lycopersicon solani*.—Department of Plant Pathology and Botany, The Connecticut Agricultural Experiment Station, New Haven.

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STERILIZATION OF AGAR MEDIA WITH PROPYLENE OXIDE

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Propylene oxide has been used as a sterilizing agent in food preservation (4), in seed treatment (1, 3), and in the preparation of plant tissue for culture media (2). The following report concerns the use of propylene oxide in the sterilization of agar media.

Difco dehydrated potato-dextrose agar (PDA) was used in all experiments. The melted agar was cooled to 45°C. To ensure contamination, a suspension of unidentified bacteria and spores of *Aspergillus* sp., *Epicoccum* sp., *Fusarium* sp., and *Trichoderma* sp. was mixed with the medium. The medium was then poured into washed unsterilized Petri plates. The sterilization chamber was a 10-L bell jar placed on a flat glass surface. To form an air-tight seal the bottom of the bell jar was coated with petroleum jelly. The lids were placed on the Petri plates, and 15 plates of media were placed in the chamber along with an open Petri plate containing 10 ml of propylene oxide.

The Petri plates were kept in the bell jar at room temperature for 24 hours. Checks consisted of Petri plates of PDA under a bell jar without propylene oxide. The plates were examined for macroscopic colonies 6 days after removal from the bell jars.

No growth developed on any of the plates from the propylene oxide treatment. All of the check plates were badly contaminated.

To determine how soon media could be used after sterilization, plates that had been sterilized with propylene oxide were inoculated with *Diplodia zeae* (Schw.) Lev., *Sclerotium cepivorum* Berk., and *Pythium debaryanum* Hesse 24 hours after removal from the bell jar. Similar transfers were made to plates containing heat-sterilized PDA. Five days after inoculation, growth of *P. debaryanum* and *S. cepivorum* in plates sterilized with propylene oxide was equal to growth in plates sterilized with heat. Growth of *D. zeae* on medium sterilized with propylene oxide was slightly inhibited.

The results suggest that propylene oxide can be used in sterilizing agar media. The method would be useful in high-school biology laboratories or at outlying experiment stations where ovens and autoclaves are not available. Even where this equipment is available, the method has advantages. Proplyene oxide probably changes many organic compounds less than does heat. It would greatly reduce the heat released in a laboratory. The lids of Petri plates do not have to be removed until inoculation, reducing the chance of contamination.

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